

Snapshot of a new industry

The much-awaited report to the US Congress on the international biotechnology industry may be flawed but it deserves to be widely read, not only in the United States.

THE US Congress, which commissioned the report on the international biotechnology industry published last week, should beware of the beguiling trap of chauvinism as it thumbs through the huge document that has now emerged (see page 402). For the Office of Technology Assessment (OTA), in the course of its patient description of who where is doing what, has unaccountably peppered an otherwise sober report with declarations that the United States has a "commanding lead" (in the development of software for the use of nucleotide-sequence data-banks) or is the "strongest in the world" (in its "efforts to commercialize biotechnology"). Few will dispute the validity of these and other assessments in the same genre, which in any case have the tactical advantage of telling Congress what it wants to hear. (Bearers of bad tidings are not much welcomed anywhere.) Moreover, these opinions bear directly on the chief conclusion of the report, that while "continuation of the initial preeminence of American companies in the commercialization of new biotechnology is not assured, . . . Japan is likely to be the leading competitor . . ."

The flaw in this jump from the particular to the general is not, of course, that it may be mistaken, for it is far too soon for anybody to tell, but that it is simplistic. OTA, which likes to be known as Congress's own think-tank, has let slip a splendid opportunity to read its masters a lesson in the economics of technological innovation and has couched its otherwise helpful answer in the outdated language of the 1950s (or even the 1930s). Indeed, the constant harping on the theme of leadership, by representing the process of technological innovation as a battle between two Goliaths, obliterates the texture of what is going on. In such an interesting field, there will be opportunities for all and, eventually, a division of labour, Adam Smith style.

Biotechnology may or may not be as old as lithofracture as a tool-making technology, but certainly dates back to the use of bacteria for turning milk solids into cheese and the use for other purposes of the liquids produced by the natural fermentation of fruit. So much, the report acknowledges in passing. In recent decades, the industry has gone up (with the use of fermentation techniques for making solvents in the 1930s) and down (with the advent of cheaper feedstocks from oil and natural gas). The novelty, in the past decade, is that techniques of genetic manipulation are certain to bring within the scope of traditional biotechnology the production of materials hitherto produced in other ways by, for example, the organic synthesis of pharmaceuticals.

Disappointment

Nobody doubts that the consequences will be important. Why else would investors in the United States and elsewhere have been so eager, in the past five years, to help launch new companies in this field? But the experience of the past five years has also shown that the new biotechnology differs in no substantial way from other new technologies, the development of steam engines in the second half of the eighteenth century for example. The immediate course of development abounds with disappointment — steam engines blow up, materials such as cloned interferon turn out not to be wonder drugs but chemicals in search of a disease to cure — yet confidence in the long-term future persists. The difficulty, in such circumstances, is that the pattern of the future is almost literally unpredictable.

The OTA report itself points to many of the potential sources of uncertainty — the likelihood that new biotechnology companies in the United States will find it harder to re-finance themselves when their original capital is exhausted, the high chance that even if things go well, many of them will have to rely on the sales organizations of established companies to ensure that their new products reach their intended users and the simple truth that vast areas of the potential of the new biotechnology are almost entirely unexplored. (Genetically manipulated plants that produce their own insecticides, for example, are a splendid and no doubt attainable goal but cannot yet be bred.) Short-term uncertainty against the background of long-term confidence should be the motto for the next few years. The question of whether the United States is "ahead", or whether Japan is a greater "threat" than any other industrialized country, is both irrelevant and dangerously misleading.

Clamour

If the new biotechnology is as important as it now seems, it will be important for decades to come and not merely for the lifetimes of the patents now being clamorously taken out. Moreover, if the opportunities for innovation span even a fraction of the ground now in prospect, there will be work enough to keep the technical communities of three continents (and perhaps even five) busy for decades to come. For the immediate future, of course, OTA is right to draw the attention of Congress to the reasons for thinking that the United States is an especially favourable place in which to embark on these exciting adventures — the availability of venture capital, the flexibility of the patent system (less is said about the problems that beset it) and the supply of talent. But the report enormously undervalues the way in which, in the long run, the emerging industry will be shaped by the unforeseeable application of unidentifiable people's ingenuity to the solution of practical problems by means that will not always yield intellectual property (as the saying goes) that can be protected.

The opportunity OTA has lost is that of reminding the US Congress that at the beginning of an industry capable of transforming the pattern of what now passes for industry, predictions of the future based on the extrapolation of the present are unsafe. What if, for example, several years of the accumulation of budget deficits on the scale made public this week should force the United States to follow other spendthrift governments along the paths that have undermined other people's entrepreneurial spirit? Or if the arrangements that have been made between industrial companies and universities turn out to be more distracting for the academics involved than the report considers likely? The danger, for the US Congress, is that while the OTA report will bring a good deal of comfort, it may also induce complacency.

What will be made of the document elsewhere? One thing is certain — it will be widely read. Governments elsewhere, but especially in Western Europe, will pay close attention to the argument that United States companies active in this field must chiefly fear competition from Japan, not Europe. On one point, they must agree with OTA: the flexibility of the system whereby new companies in the United States can be launched with the help of risk capital channelled through specialist financial companies is enviable, as is the arrangement by means of which investors may put money into specific research and development projects under-



taken by biotechnology companies in such a way as to ensure that gains are taxed as capital gains, not as income. European governments should also take to heart OTA's argument that the ease with which new companies can spring into existence in the United States, confusing though it may be for the stock markets, is a powerful spur to copious innovation and, perhaps more important, its rapid exploitation. It is, however, important to acknowledge that in the past two years, in Britain and West Germany, venture capitalism has been growing even faster than biotechnology. Whether the same governments will sanctify this trend with tax advantages comparable with those enjoyed in the United States remains to be seen.

By themselves, however, these differences do not explain why Japan should be distinguished from Western Europe as the ground in which biotechnology is more likely to flourish. Indeed, Japan (like West Germany until very recently) differs radically from the United States in that venture capital plays only a minor part in the capital financing of industry. OTA's argument is that several Japanese companies have admirable expertise in the exploitation of fermentation technology (which is true), that Japan is a huge market for pharmaceuticals (which is likely to be important in the short run) and that the Ministry of International Trade and Industry in Tokyo has launched a cooperative programme in biotechnology (which is less significant than OTA appears to think). Readers of the report will, however, conclude that OTA is most of all impressed at the number of Japanese companies that have declared their determination to prosper in biotechnology — and which are likely to turn their ambitions into reality with the zeal that has marked the success of Japanese manufacturers of automobiles and of electronic consumer goods. That is an awesome prospect but not a proof that European companies will be also-rans.

For those who would predict the future, one missing element in these calculations is the character of the relationship between the newer biotechnology companies and the larger companies with ambitions in the same field. OTA is probably right to guess that partnerships between big and small companies that already exist will often develop into even stronger relationships, outright mergers included. For large companies, which may have been slow to respond to the opportunities presented by biotechnology in the past decade, often enjoy the luxury of being able to bide their time, relying on their financial resources to be able to move quickly when the time is ripe. And once committed to the development of a new product, established companies have an advantage that newly established companies cannot but envy — an established and sometimes efficient mechanism for putting what they seek to sell into the hands of customers. Especially now that it has become plain that the benefits of biotechnology are further away than at first seemed possible, the large pharmaceutical and chemical manufacturers now operating on an international basis have powerful advantages over most newly created companies. None of this implies that there is no place for the smaller companies, nor even that none of them will grow big; indeed, OTA is entirely right to emphasize the value of the smaller companies as spurs to innovation in the past few years. But the growing importance of the multinational manufacturers in the development of biotechnology is yet another reason why pure chauvinism is out of place.

In the circumstances, the positive recommendations put forward by OTA are probably less urgent than they seem. OTA would have the administration reverse the recent decline of support for basic biological research (for which there are other good reasons) and do something unspecified to strengthen "bioprocess engineering" (for which there are few precedents). In strictly parochial terms, indeed, the threat that if Congress should fail to provide extra resources in those fields, the United States may lose its "commanding lead" to Japan or some other nation may be effective. It will be interesting to see how that issue is decided. Meanwhile, the chief value of OTA's gigantic study is that it has provided a unique snapshot of a new and important industry at a critical epoch in its growth, no longer neonate, still far short of puberty. □

Binary is divisive

Britain's system of higher education suffers from too little diversity and from being split in two.

FOR how much longer can the British system of higher education be allowed to continue as if it were two separate systems, one consisting of institutions called universities financed by central government through the University Grants Committee and the other consisting of institutions called polytechnics whose bills are paid by local authorities (with money provided by the central government)? The question has been in the air ever since the polytechnics were designated as degree-giving establishments nearly twenty years ago. The argument then was that there would be virtues in operating a dual system of higher education, if only because the newly established polytechnics could be guided, or coerced, to take a direct interest in the vocational needs of their students. It was almost as if the late Mr Anthony Crosland, the then Secretary of State for Education and himself once a university teacher, egged on by his Oxbridge civil servants, had washed his hands of the universities and decided that a quite different system would better serve the social need of higher education. In the event, the binary system (Mr Crosland's name for it) has not been much of a success. While many polytechnics have stuck admirably to their brief, and have won respect for their teaching and research in the process, others (there are twenty-six altogether) have aped the universities in the breadth of the curricula on offer, or have embraced unproved and therefore mischievous courses (admittedly fashionable in the 1960s) with elaborate titles such as "independent study", or have merely become dull. And sadly, both among academics and would-be students, the polytechnics are less eagerly sought after than are the universities.

Now, though, there is a chance that this may be changed. Guilefully, the Department of Education and Science has persuaded the universities that the system they operate for administering applications to enter universities from school students should also be used to administer applications for entry to the polytechnics. The scheme (whose cost will be met by the government) will operate for a trial period of two years, but everybody concerned insists that students wishing to hedge their bets on securing a place somewhere in higher education will have to complete two separate application forms, the data on which will be sedulously kept separate. This is either a nonsense or the thin end of a wedge, one that leads to a unified procedure for entry into higher education of either kind.

And not before time. Not merely young people looking for places in higher education but those who eventually employ them are thoroughly confused by the present system, which is a disgraceful monument to the corrosiveness of academic snobbery. Both types of institutions allow young and not-so-young people to earn degrees, whose authenticity in the case of the polytechnics is vouched for by an independent council. Polytechnics, it is true, lack people called vice-chancellors, and have directors instead. Senior teachers are called "heads of department", not professors. And they have courses in hotel management and catering as well as in Latin and Greek. But could all British universities justly claim that they are themselves so very different?

If not, university academics should follow the only honourable course open to them, and clamour for the abolition of the binary system by means of the abolition of the boundary between its two unjustly unequal parts. The opportunity will arise when they come to reply to the questionnaire put out last autumn by the still-new chairman of the University Grants Committee, enquiring in a neutral fashion about university opinion on the binary system. The practical benefits are obvious — students will no longer be quite as mystified, while more efficient use will be made of scarce resources. More important, however, the British system of higher education would at one swoop acquire the diversity of institutions only now being forced to emerge by financial deprivation. □

US defence research

Pentagon asks for stricter control of publication

Washington

THE Pentagon is seeking extensive new powers to change or block the publication of scientific papers written under Department of Defense (DoD) contracts. In a new policy directive (number 2040.2), DoD sets out a general policy for reviewing both basic and applied research carried out in academic settings.

Under the scheme, basic and applied research papers deemed not to be sensitive will have to be submitted to a DoD contract office at the same time as they are submitted for publication, although DoD will have no right to insist on changes or to block publication. Basic research characterized as sensitive will have to be submitted to DoD 60 days before it is submitted for publication. In these cases DoD will be allowed to suggest changes or recommend that publication should not go ahead, but the final decision will rest with the investigator.

Applied research papers which are sensitive will, however, have to be submitted to DoD 90 days before they are

The restrictions on publication proposed in the DoD directive are far stricter than those recommended by the academy's report, prepared under the direction of Dale Corson, former president of Cornell University. The main conclusion of the Corson panel was that too much secrecy in scientific communication would damage rather than strengthen US security because of its chilling effect on the scientific enterprise itself.

Warning that the effort to protect sensitive information was spread too thinly, the Corson panel proposed confining controls to a few "grey areas" where the technology was developing rapidly, had clear military applications and would, if released, give the Soviet Union a short-term military advantage it could not otherwise acquire.

DoD, however, wants to extend the scope of its controls. It suggests a looser definition of sensitive technology as that "perceived to have a military impact" and in which the Soviet Union is less advanced than the United States. Furthermore, DoD wants a new Militarily Significant Emergent Technologies Awareness List (METAL) to pinpoint frontier technologies that could be candidates for stricter controls.

DoD's break with the Corson recommendations has come in spite of a formal improvement in liaison with the uni-

versities. Twenty academics have been invited to join an academic advisory group for Comex, the committee on exchanges which recommends whether scientific visitors from unfriendly nations should be granted visas. A government/industry/university research round table has been established within the National Academy of Sciences but has not yet met. And a working group on export controls, attached to the DoD/University Forum, has given the academic community periodic progress reports on the Pentagon's internal review of scientific controls.

The complexity of the existing control mechanisms continues, however, to baffle both scientists and officials. For example, an extension course on materials science held recently at the University of California, Los Angeles (UCLA), was barred to foreign citizens. Michael Bley, UCLA's director of marketing, said some of the technologies to be discussed were on the US Munitions List. But he admitted that publicity for the course had not mentioned that citizens of the United Kingdom, Canada, Australia and New Zealand would have been able to attend under the terms of a technical cooperation agreement with the United States.

Publication, if it is ever declassified, of the interagency study on technology transfer may clarify the issue. The DoD study, however, gives little cause for optimism. From the point of view of the universities, its only welcome recommendation is the creation, for the first time, of a technology transfer appeals board within the Pentagon. Otherwise, its recommendations are far more conservative than Carson had wished.

Peter David



submitted for publication, and DoD will be entitled to insist on changes in the manuscripts or to block publication altogether.

The new directive is part of an extensive Pentagon review of technology transfer which started in 1981 and is now nearing completion. As part of the review, six panels looked at research contracts, visa controls, emerging technologies, scientific conferences, publication and rules for exemption from the Freedom of Information Act. Their findings are being coordinated with those of a still secret interagency review of technology transfer started in 1982 after a National Academy of Sciences report called for an easing and simplification of existing controls on the publication of research with a bearing on national security.

Ariane launch delay

Europe's hands clean

THE launch of Ariane, Europe's hope of an answer to the space shuttle, is suffering from greater and greater delays, just as the shuttle is getting into its stride.

For once, however, the problem is in the United States. Ford Aerospace in Palo Alto, California, is desperately trying to remedy a fault discovered in the Intelsat V (F8) series of communications satellites, one of which was due to have been launched by Ariane last November. Neither Ford nor the European Space Agency (ESA), administratively responsible for launching the satellite from its Kourou (French Guyana) base, will commit itself to a date more precise than "the end of February". Only two weeks ago, the canvassed launch date was mid-February.

The problem — interference on an L-band maritime communications system attached to Intelsat Vs from the fifth onwards — is "intermittent", says Ford. This may be taken as a code word for almost impossible to reproduce.

The Intelsat organization has leased these L-band systems to London-based Inmarsat, which deals solely in ship communications, and Intelsat will suffer penalties if the systems do not work. Equally, however, the organization faces penalty clauses in the contract with ESA, if launch is delayed too long. But, according to an ESA official, relations with Intelsat have not deteriorated to that stage yet: "we are not thinking about it", said the official.

According to ESA, if Intelsat V (F8) does get away by the end of February, there will still be five Ariane launches this year. But this compares with as many as eight foreseen last May and with ten scheduled shuttle launches. The next 18 or so satellites to be launched in the Ariane series, some in multiple launches with the more powerful Ariane-3 launcher, are all communications satellites, the first novelty coming with the high-resolution French SPOT remote sensing satellite, now due to be launched some time in mid-1985.

Robert Walgate

OTA biotechnology report

Congress told US ahead, Japan second, rest nowhere

Washington

PREEMINENCE in the life sciences, abundant venture capital and an entrepreneurial spirit have combined to give the United States a commanding lead in the commercial application of new biotechnological techniques, although Japan will pose a growing challenge. That is the conclusion of a 612-page study of the international biotechnology industry published last week by the Office of Technology Assessment (OTA), Congress's science think tank.

The report, *Commercial Biotechnology: An International Analysis*, predicts that within the next 5–10 years a range of new biotechnological products — including pharmaceuticals, animal vaccines and speciality chemicals — will reach the market. Europe is lagging behind the United States and Japan but a few big chemical and pharmaceutical companies in Britain, France, West Germany and Switzerland may compete strongly in the manufacture of some products.

OTA ascribes the US lead to a number of factors, notably its uniquely favourable tax climate. In 1983 alone, the private sector invested more than \$1,000 million in the commercialization of biotechnology in the United States, and since 1976 more than 100 new biotechnology companies have been started up with private venture capital. Underpinning this effort is an enormous investment by the federal government in basic research in biotechnology — some \$500 million a year compared with about \$60 million each by Japan, Britain, France and West Germany.

Ready access to venture capital has given the biotechnology industry in the United States a different structure from that of other countries. While its main competitors have depended for the commercialization of biotechnology on established companies, the US effort has been strengthened by a profusion of embryonic companies that have sharpened competition and hastened the spread of expertise from the universities.

But the US lead is not unassailable. OTA believes Japan could catch up if the United States continues to prune funds for basic research in the life sciences and to neglect applied research and bioprocess engineering — the skills that take novel production techniques the laboratory to the factory. Japan has more bioprocess engineers than the United States and spends a bigger slice of its science budget on the solution of applied research problems. Its knack of beating other countries in the race to apply their own basic research could, says OTA, give Japan a bigger share of the biotechnology market than the United States.

What accounts for the United States' present lead? The report says the three most important factors in the competition to commercialize biotechnology are the financial and tax incentives provided by governments, the amount they invest in research and the degree to which they ensure that there are enough well trained experts. In all three areas the United States scores higher marks than its competitors but it cannot afford to be complacent.

The big postwar investment in the basic life sciences has given the United States a competitive edge in the supply of molecular biologists and immunologists, but there may not be enough plant molecular biologists and scale-up experts. Japan, Britain and West Germany, unlike the United States, have invested steadily in generic applied microbiology and bioprocess engineering, areas which will become increasingly important as the industry turns from its present fascination with research and development towards the manufacture of products.

OTA also adds a warning that the dynamism of the newly created biotechnology companies cannot be taken for granted as the industry matures. The existing division of labour between the small companies that have pushed ahead fastest in research and the established companies that have concentrated on marketing and production may break down as the larger companies do more of their own research. And many of the new companies may be unable to raise the money they will need in 5–10 years to move from research to production.

OTA expects the real test of US competitiveness to come when large-scale production begins and bioprocess problems must be faced, initially in pharmaceutical markets. Here the United States will face impressive competition from both Japan and Europe. OTA notes that Britain's major pharmaceutical companies — such as ICI, Glaxo and Wellcome — have a great deal of expertise in bioprocessing and that Britain conducts some of the strongest basic research in interdisciplinary plant sciences.

Japanese companies, spurred by government "targeting" of biotechnology, have launched a new drive to enter international pharmaceutical markets. Japanese companies are already world leaders in large-scale plant tissue culture, and the Ministry of International Trade and Industry (MITI) has identified secondary compound synthesis from plants as a major area for commercialization. Unlike the United States, Japanese industry is investing heavily in research on speciality chemical production, an area where Japan is already prominent.

Peter David

Europe

OTA's slight resented

THE Office of Technology Assessment (OTA) report on world biotechnology seriously underestimates Europe's potential, specialists at the European Commission in Brussels were saying last week. "The report puts Europe firmly in third place, with Japan the only competition for the United States", but this overlooks the quality of basic research in Europe according to one official.

Another critic, Dr Mark Cantley, who for two years headed a team investigating the future of biotechnology in Europe as part of the Commission's FAST (Forecasting and Assessment in Science and Technology) investigation, and who is listed as a contributor to the report, says that the last version of the OTA report that he saw was "a relatively messy document", uneven in quality.

A sense of umbrage about the report has been building up in Europe since nearly a year ago, when drafts of the final version first became available. It seems that many European governments were able to persuade OTA to incorporate last minute assessments of the state of biotechnology in their countries. The difficulty seems to have been OTA's reliance on published documents and reports from national contributors rather than on first-hand investigation.

Cantley nevertheless says that OTA is correct in its political judgement of the "difficulty of getting our act together". He illustrates this with the problem of isoglucose in Europe, where a cheap enzymatic method for producing the sugar was effectively vetoed by the sugar-beet lobby. One of Cantley's hopes for the future is that it may be possible to bring about a better integration of agricultural and industrial policy so as to remove impediments to innovation in the agricultural field.

One of Cantley's colleagues, Ken Seargent, noting OTA's dismissiveness of Europe, nevertheless insists that "it need not be like that". He cited both the potential of research in the natural sciences and practical successes such as the pyrethrin analogue insecticide developed in Britain.

Meanwhile, in Brussels, officials were hoping earlier this week that the Commission would agree on Wednesday 2 February to establish a "concertation unit" in biotechnology as a catalyst for more European cooperation in the field. Research commissioner Etienne Davignon is thought to consider this step so important that he will find the money (roughly \$1 million a year) from within his existing budget, thus avoiding reference to the strife-torn Council of Ministers.

Some in Brussels even consider the new unit as the foundation stone of a European

policy on biotechnology as ambitious as the information technology policy embodied in the Esprit programme still awaiting political approval and financial blessing. In the meantime, however, the

concertation unit would establish and maintain a network of experts, strengthening the present arrangements for coordination on biotechnology among the ten member states.

Robert Walgate

Finance

Small companies star

ONE theme running through the Office of Technology Assessment (OTA) report is the importance to US biotechnology of the newly formed specialist companies of which more than 200 are listed in the report. OTA says that these appear to be more productive of research-based innovation than established companies when compared person for person, and that they have a tendency to bring research results to the market more quickly than better established companies. The report notes, however, that newly formed companies, launched with private funds or by means of a public offering, may find the problems of refinancing more difficult when their initial capital is used up, if only because investors in new companies necessarily look for good returns. This opinion appears to have been written before the onset of the fall in the value of biotechnology stocks last autumn.

In an analysis of 18 US biotechnology companies whose shares are publicly traded, OTA points out that only three (Cetus, Genentech and International Genetic Engineering) had positive earnings in the latest complete financial year and that two of these were in that happy position because of interest earned from funds obtained in public offerings. OTA notes that the high cost of financing research programmes has persuaded many companies to finance their operations through research contracts with larger companies or to recruit capital by means of limited partnerships in respect of research and development projects. For similar reasons many companies are giving priority to the development of products that will have an immediate market, such as monoclonal antibodies for the diagnosis of disease and veterinary products and feed additives for livestock.

In spite of the publicity given to biotechnology, OTA points out that venture capital is still flowing more strongly into the fields of communications, computers and electronics, which consumed nearly 60 per cent of investments in 1982. One striking feature of the pattern of investment in the past few years has, however, been the willingness of established US companies to invest in their smaller brethren. In 1982, the total investment in new biotechnology companies from this source is estimated to have been \$120 million but to have fallen to \$40 million in 1983, chiefly because of the increasing attention paid by the larger companies to strengthening their own in-house activities.

Looking ahead, OTA says that new companies may ultimately suffer from these ar-

rangements because of the low profit margins to be expected from patent royalties, the chance that research contracts will not be renewed and the willingness of the larger partners in research contracts to devote resources to the exploitation and marketing of new products. For these reasons, OTA concludes, successful biotechnology companies will in due course find it necessary to engage in the whole range of activities bearing on the exploitation of their innovation.

On the links between universities and industrial companies, new or well established, OTA notes that there are advantages for both partners as well for the "national innovative process". At the same time, it points out that universities cannot expect that royalty income from patents will ever be a substantial part of their current costs. On the basis of a questionnaire sent out to university administrators, teachers and students, OTA comes to the conclusion that the reduction of federal funds for universities has been a spur, that few difficulties have arisen in the conduct of these relationships, and that 85 per cent of respondents considered that links with industry had not damaged university research. □

West Germany

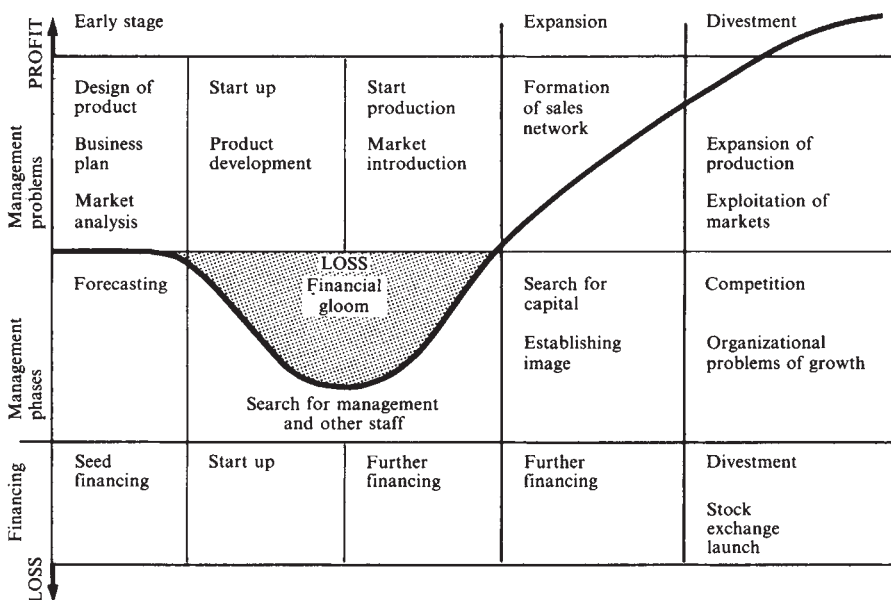
More adventure from capital

OBSTACLES to technology transfer and the formation of new companies in West Germany could be swiftly removed if a motion tabled in the Bundestag by two parties of the government coalition (the Christian Democratic Union, CDU, and the Free Democratic Party, FDP) is adopted. The need to improve the position of small and medium-sized companies in the Federal Republic is confirmed by the dim view of European investment in biotechnology taken by the US Office of Technology Assessment (OTA) (see page 402).

Although the stock market recently reached an all-time high and West Germany is still a leading world exporter, its share of world trade has fallen. Except for a few traditional areas such as chemicals, automobiles and electronics, the country has become an importer of high technology. More companies are going bankrupt than are starting up whence the interest in the search for means to encourage the formation of new high technology companies.

A major part of the problem is recognized to be the dependence of West German industry on loan capital rather than equity (risk) capital, which at present accounts for only 20 per cent of the capital employed by public companies, with a mere 5 per cent representing savings by members of the public. The banks, the main source of capital and the only insti-

Profile of a company start-up by venture capital



VENTURE capitalism combines financing with management and technical expertise. The investors take an active role in the development of the company. Misjudgement, takeover, merger or loss of crucial staff are a few "risks" that would give a different profile. A successful investor can hope to see this picture emerge for one third of his ventures; one third would level off to a low profit; one third would be written off. A reasonable time would be five years. □

tutions allowed to launch companies on the stock market, have been reluctant to support ideas and technological expertise alone, preferring more tangible bricks-and-mortar propositions. In addition, high taxes and charges discourage companies from flotation on the stock market and make equity capital more expensive than bank finance. It is estimated that this has created a DM100,000 (£25,000 million) "equity gap". In 1984, some 600 new companies will probably be launched on the US stock exchanges, whereas in West Germany, if the present situation continues, there will probably be no more than 20 new arrivals.

The lack of venture capital, the power behind the technology transfer represented by Silicon Valley and US biotechnology, is acute in West Germany. Yet in the United States since 1970, venture capitalists in 600 firms have invested a cumulative total of more than \$8,000 million and have, in the process, created more than 600,000 jobs. Fifteen per cent of this capital has come from abroad and an optimistic estimate is that some DM 100,000 million could be available in West Germany if the right investment framework were created. Citicorp, experienced venture capitalist of the New York Citibank, is planning to invest DM420 million in Europe, including DM 140 million in West Germany.

Home-grown steps in this direction have also now become apparent. Apart from the Wagnisfinanzierungs GmbH, founded by a grouping of 29 banks, the most significant venture fund in West Germany has been started by Siemens under the name Techno Venture. The fund is capitalized at

DM130 million, of which Siemens has contributed DM20 million and DM70 million has come from within West Germany. Private groups administer other funds, a small community has launched a fund through its local savings bank and the city of Berlin has begun a DM10 million fund. Minister of Research and Technology, Dr Heinz Riesenhuber, himself a successful businessman has advocated that 0.1 per cent of institutional funds should be used as venture capital.

Dr Riesenhuber emphasizes the need to create the right framework for research, technology transfer and commercialization, rather than direct state support and direction.

In the United States last year, it is estimated that 32 per cent of venture capital funds were contributed by pension funds, 32 per cent by insurance companies and 21 per cent by industry and foundations. Other financial routes such as tax-favoured research and development partnerships also exist to channel private funds into US industrial growth points. The OTA report concludes that "the unique complementarities between established and new firms, the well-developed science base, the availability of finances and an entrepreneurial spirit have been important in giving the United States its present competitive advantage in the commercialization of biotechnology . . . the (European countries) lag behind because they generally do not promote risk-taking, either industrially or in their government policies." The CDU/FDP motion is one step towards correcting this.

Sarah Tooze

Technical innovation

Plea for UK tax-breaks

THE British treatment of scientific research and patenting costs for taxation purposes is anomalous and may be inhibiting research and innovation in Britain, according to a report published last week by the Technical Change Centre. The report, written by a former senior economic adviser at the Inland Revenue, concludes that there is a powerful case for a more favourable tax treatment of research, which would be helpful to progressive industrial companies and would cost no more than £80 million in its first year.

The Technical Change Centre, which is supported by the Leverhulme Foundation, the Science and Engineering Research Council and the Economic and Social Research Council, produced studies on a variety of subjects, linked by the common theme of technical change. Its examination of the tax treatment of intellectual property in Britain finds that Britain gives highly favourable treatment to capital expenditure on plant and machinery compared with that on patents and know-how. As

things stand, capital expenditure on patents in Britain is written off over 17 years and expenditure on know-how over 6 years. The specific proposal examined is that expenditure should be written off as it is incurred, like that on plant and machinery. This would, it is argued, restore some of the advantages once enjoyed by research that have now all but disappeared.

The report considers a number of possible objections but does not find any of them conclusive. Some, like the probable need for more civil servants in the Patent Office to cope with the likely increased number of patent applications, are dismissed as only "debating points", while others, such as the objection that companies might make increased use of patents as marketing gimmicks, are considered to be small risks.

On the positive side, it is concluded that the total cost of the scheme would be justified to the public by its immediate benefit to the more technologically progressive firms that are thought to be essential for Britain's future economic prosperity. While the cash flow of industry as a whole would not be affected to any significant degree, there would be a strong incentive to licensees to acquire patents if their costs could be written off immediately.

Tim Beardsley

China's research

Call to go back to basics

CHINA'S minister in charge of the State Scientific and Technological Commission, Mr Fang Yi, has spoken out firmly in favour of basic research as the foundation of the future scientific and technological development of the country. Addressing the fifth session of the scientific council of the Chinese Academy of Sciences recently, Mr Fang Yi noted that because major technological advances will become more and more dependent on breakthroughs in basic research, "some foreign countries" will be less willing to publicize their basic research in the open literature. China should therefore pay ever more attention to fields of basic research that will "open new paths" for technological development.

Mr Fang Yi's remarks differed considerably in emphasis from other recent official statements which put greater stress on the importance of applied research for the development of the Chinese economy, although he did note that particular emphasis should be given to "those fields of basic research which could have significant effects on the long-range development of the national economy". Current guidelines specify these fields as information science and technology, biotechnology, lasers, nuclear technology, computers and marine engineering.

As far as "big science" is concerned, with its demands for "enormous human, material and financial resources", Mr Fang Yi said that China's "limited conditions" imposed a need to be selective and to carry out only a few projects "according to our capabilities". Once a project is approved, however, scientists should be left to work in peace, since basic research demands "long-term efforts" and a tranquil atmosphere in which scientists can work in a "steady and sustained manner".

Mr Fang Yi's remarks seem intended as a not-too-veiled criticism of the attitude which still prevails in China as an aftermath of the cultural revolution and which is highly critical of the value to the economy of "intellectual" work. (Cases have been reported during the past year of misguided party activists criticizing the payment of bonuses of a few hundred yen to engineers and designers whose innovations have produced a very much larger financial gain.)

At the same time, even the Chinese Academy of Sciences will not have an entirely free hand. It must, Mr Fang Yi said, "show an adequate division of work and foster close cooperation with industrial departments, universities, national defence research departments and local research centres". In the applied sector, the Academy of Sciences must carry out research "more farsightedly, sooner and more intensively" than these other bodies.

Vera Rich

Netherlands energy policy

More wind in prospect

Waalre, The Netherlands

AFTER two years, during which some 42,000 people took part in discussions throughout the country, great public debate in the Netherlands on energy policy has finally ended. But for the politicians, for some of whom the debate has conveniently postponed difficult decisions, the argument is only now beginning.

Predictably, the consensus reached in the final report on the debate favours much greater efforts in conservation and greater use of renewable energy. There is a need for the continued use of coal, the report concedes, but it comes out against the idea of any new nuclear power stations.

About Dfl 28 million (£6.3 million) has been spent on the two-year consultation exercise, of which Dfl 8.5 million went on 63 information projects (brochures, exhibitions and so on) and 14 research projects (energy analysis of nuclear power, for instance). A nine-member steering committee was set up in July 1981 under the chairmanship of the then minister for higher education and science policy, M. de Brauw. The committee had a scientific staff of 14 and an administrative staff of 16.

The idea for a public debate on energy came originally from the largest of the Protestant churches, with support from the largest labour union. In 1980, a parliamentary decision was made to embark on the public debate, providing a forum for the heated discussions that followed the abandonment in 1976 of a plan to build three nuclear power plants. Ironically, that plan had been put forward by Ruud Lub-

bers, then minister for economic affairs, who is now prime minister.

The first reactions to the report from the electricity producers are hostile. Public feeling against new power plants using fossil fuels is sustained by the knowledge that at present the Netherlands has a 50 per cent surplus of electricity generating capacity (the total is now 16,000 MW). After 1992, however, new capacity will probably be needed to replace obsolete plant. The report calculates that a "decentralized capacity" of 8,000 MW is feasible — 2,500 MW as wind power and 5,500 MW as combined heat/power in industry and city heating systems. In the past few weeks the electricity producers have been lobbying for

more nuclear power, but to little avail.

In condemning nuclear power, the report stressed the problem of disposal of nuclear waste in a country as densely populated as the Netherlands. Supporters of nuclear power have concentrated on the high cost of electricity in the Netherlands compared with that in neighbouring countries such as West Germany and France. The steering committee claims, however that costs in France are kept artificially low by large government subsidies.

Environmental groups have naturally welcomed the report's conclusions, but whether any action is taken will depend on the politicians. And as the present policies of the three main political parties are not in line with the outcome of the public discussion, it may be a long time before Dutch energy plans for the 1990s are known.

Casper Schuurung

Polish science

Academy's position compromised

THE Polish Academy of Sciences has experienced some difficulty in the election of a new president, and many academy members see this as an indication of increased government and party supervision of the academy. Unlike most socialist countries, Poland has no effective analogue of the Soviet State Committee for Science and Technology, so the academy plays a major role not only in basic science but also in the coordination of applied research. The academic secretary of the academy holds ministerial rank, and reports directly to the prime minister. During the liberalization of 1980–81, there was pressure within the academy to make the academic secretary responsible to the academy members.

The promised new legislation on the academy is said still to be in the preparation stage, although it will almost certainly differ considerably from the hopes of the Solidarity period. In the meantime, Dr Aleksander Gieysztor, a historian, who was elected president in the early days of Solidarity, came to the end of his three year appointment, and was due to retire or stand for re-election in December. Party officials responsible for scientific matters thought Dr Gieysztor's reelection would be a hindrance to closer ties between the Polish academy and the academies of other "friendly" (that is socialist) countries, and he was urged not to stand for re-election.

The party's own choice for president was Dr Leonard Sosnowski, a solid state physicist, who is personally popular with many academy members. In the first round of academy elections in December, however, Dr Sosnowski was roundly defeated in the secret ballot. It seems likely that some voted against him on the grounds that they did not wish to be "manipulated" by the party, while others felt that, without casting any aspersions on Dr Sosnowski himself, it was unwise on principle to elect as president a

party member, because this might give rise to a conflict of interests if the academy found itself opposed to party policy.

The second round of voting last week, however, resulted in a clear winner, Dr Jan Karol Kostrzewski, an epidemiologist and a vice president of the academy, of whom little appears to be known — except that he is not a party member.

Vera Rich

Spare parts wanted



THE California Academy of Sciences in San Francisco is appealing for help in its search for spare parts for the 6.4 inch telescope installed in 1879 at what was then the city's first observatory. Access to a similar telescope would allow the missing parts to be replicated.

The instrument was constructed by the Fauth Company and the mounting may even have been the first of that company's model 72 to have been built. Those who know of the whereabouts of a telescope of this construction are asked please to contact Allan Wilson at the California Academy, Golden Gate Park, San Francisco, California 94118. Collect calls will be taken. □

It's an ill wind. . .

THE following exchange took place in the British House of Lords on 23 January. Lords Paget and Cledwyn are members of the Labour Party, Lord Trefgarne is an under secretary of state in the Ministry of Defence.

Lord Paget of Northampton: My Lords, does not the noble Lord the Minister feel that all these questions are along the wrong lines? *Homo sapiens* has launched upon a career in which he is numerically doubling himself every 30 or 40 years. He is plainly in need of a massive cull. Nuclear weapons will make possible that massive cull — and, even more, they have the special factor of muddling up the genes so that from the new crop, something rather better than the present lot may emerge. Ought we not look to our good fortune?

Lord Trefgarne: My Lords, the originality of the noble Lord's views never ceases to impress me.

Lord Cledwyn of Penrhos: My Lords, is the noble Lord the Minister aware that what my noble friend has just said is not Labour Party policy? □

Genetic manipulation

British watchdog reconstituted

BRITAIN'S Genetic Manipulation Advisory Group, known as GMAG (pronounced Gee-mag with the first syllable as in "genome"), met for the last time last week. The group is to be replaced by a new body, the Advisory Committee on Genetic Manipulation (ACGM), which will give advice on safety aspects of recombinant DNA techniques to the Health and Safety Commission, an independent body charged with devising policy on occupational safety for implementation by the Health and Safety Executive.

The formation of ACGM was announced in the House of Commons by Mr Peter Brooke, Under Secretary of State in the Department of Education and Science, under whose aegis GMAG operated. Like GMAG, the new body will have representatives of employers' and employees' organizations as well as technical experts. Unlike GMAG, however, ACGM will have no representatives of "the public interest". The new committee, whose membership has not yet been announced, will be concerned with policy matters, leaving the scrutiny of individual experiments to officials.

There has been a growing consensus that work involving genetic manipulation holds fewer hazards than was at one time feared, and so GMAG's role has become correspondingly less important. It would have been disbanded before now but for the delay in setting up a parallel body, the Advisory Committee on Dangerous Pathogens, itself caused chiefly by an outbreak of smallpox infection at the University of Birmingham in 1978. The government seems to have decided that it would be politically inappropriate to do away with GMAG while the pathogens committee was being reorganized.

ACGM will take more of a back seat than did GMAG. GMAG members, many of whom will serve on the new committee, have, however, been told that the new committee will be free to initiate action and to plan its own work. The decision to hand over the day-to-day business of keeping records of recombinant DNA research to the Health and Safety Executive was fully approved by GMAG members, although some working scientists doubt whether the change will make their lives easier. Most say they were content with the way in which GMAG carried out its duties in recent years, since the levels of containment required for most work have been reduced and notifications of routine work can now be made retrospectively. In future, scientists will have to deal with officials of the Health and Safety Executive who may be less easy to please than GMAG.

The new committee has been given by GMAG a list of several urgent topics for discussion, including commercial con-

fidentiality, the release of genetically engineered organisms into the environment and work with oncogenes. Despite industrial scepticism at the outset, GMAG seems to have been able to assure commercial companies of confidentiality; now, doubts have arisen whether the constitution of the Health and Safety Executive, which includes provision for the disclosure to work-people of companies' plans, will allow this guarantee to be continued.

British opinion on the release of genetically-engineered organisms into the environment, recently a source of controversy in the United States (see *Nature* 305, 564; 1983), is likely first to be tested by research at the John Innes Institute in Norwich. Work with *Rhizobium* bacteria, which fix nitrogen in legumes, holds the promise of allowing the host specificity of different species to be altered, when the efficiency of nitrogen fixation might also be

increased. Before approving field trials, ACGM will have to be sure that an experimental organism could not compete with wild types or, alternatively, could be destroyed if necessary. One fear is that host growth could be inhibited: disablement of legumes with a selected strain of *Rhizobium* has already been demonstrated and an engineered strain could conceivably be an effective pathogen. And the committee is asked to note the growing evidence that some human oncogenes linked to particular promoters may function like animal tumour viruses.

While nobody doubts the competence of the new committee to tackle these issues, another question remains: who will be responsible for considering ethical problems that may arise with genetic manipulation techniques? The government's Warnock committee, which is expected to report in the summer, is considering these questions as they relate to human fertility, but some members of GMAG want to see more permanent provision made. The issue is so far unresolved.

Tim Beardsley

European air pollution

UK inspector protests at Brussels

SHOULD air pollution controls in Britain be tightened in order to combat acid precipitation? The latest body to enter the debate is the Industrial Air Pollution Inspectorate. In the inspectorate's annual report published this week*, the chief inspector, Dr Leslie Reed, inveighs against the use in legislation of emission limits such as those planned in two European Commission proposals for Community directives. Dr Reed will be giving evidence on the European air pollution proposals next week to the House of Lords' European Communities committee.

The cornerstone of British air pollution control since the first Alkali Act was passed in 1863 has been the statutory requirement that operators of scheduled plant should employ the "best practicable means" to control emissions. The chief industrial air pollution inspector, who was until recently known as the chief alkali inspector, has in practice drawn up presumptive emission limits — specified as milligrams of pollutant per cubic metre of air emitted — but these are used to indicate only whether an operator is making a reasonable effort and have no force in law. Dr Reed's defence of the system is that presumptive limits can be changed rapidly as technological advances allow and that the actual emissions allowed may be altered to suit local circumstances.

Britain has already objected at Brussels to the section of a proposed framework directive on air pollution control that calls for the introduction of Community-wide emission limits agreed by a two-thirds majority. The European Communities' Council of Ministers will meet in March to try and find a way round the impasse. A

second proposed directive on fixed emission limits for major combustion plant, which also specifies targets for swingeing cuts in total national emissions, is likely to be opposed by Britain. This proposal is being pushed hard by West Germany which fears that transboundary pollution is contributing to mounting damage to its forests.

But Dr Reed fears that adopting these directives will introduce another tier of authority in Britain for the sake of "one or two European countries which have inadequate controls of their own". Dr Reed also has doubts about a third European directive on air quality standards for nitrogen dioxide. While accepting the principle, Britain should, he says, resist demands for standards that would require costly measures by industry to ensure compliance but which are based on inadequate evidence of need.

Acid rain was the subject of a major speech recently by Mr Ian MacGregor, chairman of the National Coal Board, who said that "after we have crippled some of our basic industries we could then find we had not solved the problem". He pointed out that volcanic activity may emit huge quantities of sulphuric acid into the atmosphere and that some of the reported damage to conifers was unlikely to be due to sulphur dioxide pollution because the trees bore healthy growths of lichen. He concluded that short-term action, such as liming affected waters, should be applied vigorously until science is able to establish the facts.

Tim Beardsley

*Industrial Air Pollution: Health and Safety 1982 (Health and Safety Executive).

Wolf winners

Rehovot

THE winners of the 1983–84 Wolf Foundation Prizes in the sciences have been announced in Herzliya, Israel. The Wolf Foundation was set up in 1976 with an endowment fund of \$10 million and the prizes, worth \$100,000 each, have become known unofficially as the Israeli Nobel prizes. The prizes are awarded for chemistry, physics, mathematics, agriculture, medicine and the arts, but this year there will be no prize for medicine. (The winner of the arts (architecture) prize has yet to be announced.)

The chemistry prize is shared by three US university professors, Herbert S. Gutowsky of the University of Illinois, Herden M. McConnell of Stanford University and John S. Waugh of Massachusetts Institute of Technology, for the "singular contribution" of magnetic resonance spectroscopy to the theory, structure and dynamics of molecules.

The physics prize is shared by Professor Erwin L. Hahn of the University of California at Berkeley, Dr Theodore H.



Sir Peter Hirsch — a share in the physics prize

Maiman of TRW Inc., Los Angeles, and Sir Peter Hirsch, chairman of the United Kingdom Atomic Energy Authority and professor of metallurgy at the University of Oxford. They are honoured for their work in discovering nuclear spin echoes, for the realization of the first operating laser, and for the development of the scanning electron microscope respectively.

The mathematics prize is shared by Professor Shiing S. Chern of the University of California at Berkeley and Professor Paul Erdos of the Mathematics Institute at the Hungarian Academy of Sciences in Budapest. Both are described as "veteran mathematical giants". The agriculture prize goes to Professor Don Kirkham of Iowa State University and Professor Cornelis T. de Wit of the Agricultural University of Wageningen in the Netherlands, both of whom have made "innovative contributions" to the quantitative understanding of soil/water and other environmental interactions influencing crop growth and yield. The awards will be made in Israel in May.

Nechemia Meyers

US space station

NASA seeks partners, purpose

Washington

WITH presidential approval for a space station tucked firmly under his belt, James Beggs, administrator of the National Aeronautics and Space Administration (NASA), is about to tour world capitals in search of international partners for the new venture. And because NASA has still only the sketchiest notion of what it wants from a space station, countries willing to make an early investment can expect a big say in the final design.

During the past two years, while NASA has been arguing the case for a space station, tentative discussions have taken place with the European Space Agency, a number of individual European nations, Canada and Japan. But until last week, when President Reagan gave the space station formal approval in his state of the union message, NASA had been unable to negotiate in earnest.

Beggs is now in an unusually strong position. His status during his overseas tour will be as President Reagan's personal emissary. Meanwhile, one of the principal impediments to foreign collaboration — the involvement of the Pentagon — has been removed. The Department of Defense has so far refused to find a military argument for supporting the new venture. As a result, the space station is being justified by the administration solely on scientific and commercial grounds, and there will be no Pentagon contribution to the \$8,000 million development budget.

For overseas partners, however, the most attractive feature of NASA's space station is that it has not yet been designed. Beggs intends to wait for at least another two years before completing plans for the station and beginning hardware development. After the state of the union message last week, NASA was careful to keep the technical specifications of the space station as vague as possible.

All that NASA is saying for the moment is that the station will provide for a permanently manned base in a low inclination orbit. In addition to living quarters for a crew of six to eight, the station will house data-processing facilities and a docking hub for the shuttle. Pressurized modules for scientists and astronauts will be attached to the station, as well as unpresurized free-flying platforms capable of performing a variety of functions.

The purpose of the space station is, so far, almost as vague as its design. Possible roles listed by NASA range from the relatively mundane, such as the tending and repair of satellites and space telescopes, to the frankly exotic. In the long run, NASA maintains, the space station could become the first step towards the establishment of a manned lunar base, or a manned mission to Mars.

Despite its glee at having at last won its

battle for a space station, NASA was careful last week to mollify some of the groups that had opposed it. The space science board of the National Academy of Sciences concluded last year that developing a manned space station could not be justified on scientific grounds alone. NASA is now promising to work closely with the space science community and to do its best to ensure that space science funds are not raided to provide funds for development of the space station.

Peter David

US materials science

Setback for Keyworth

Washington

AFTER a year of trouble for his plan to establish a National Center for Advanced Materials (NCAM) at Lawrence Berkeley Laboratory (see *Nature* 301, 456; 1982), presidential science adviser George Keyworth has bowed to the inevitable and asked the National Academy of Sciences to study priorities for "major facilities" for materials science.

The original NCAM plan met with harsh criticism from many materials scientists who complained that the proposal had been sprung on them without warning and without the benefit of peer review. Responding to these criticisms (and perhaps sensing a budget item that could be raided for several pork-barrel projects), Congress last spring cut the \$26 million that the President had proposed for NCAM in his budget for fiscal year 1984 to a mere \$3 million and ordered that no more should be spent until a scientific review of the project was completed.

A Department of Energy panel headed by Albert Narath of Sandia National Laboratories was appointed to carry out this review; last fall it declared that the centrepiece of the NCAM proposal, a new advanced synchrotron light source intended as a national facility should not be locked up in a laboratory dedicated to materials research. NCAM has since become mere CAM, and Keyworth's efforts to turn the spotlight on materials science have been pushed back to square one.

The National Academy panel, headed by Frederick Seitz, president of Rockefeller University, New York until 1978, will be considering facilities with an initial cost of \$5–10 million or more, including synchrotron and neutron sources. The panel will report next summer, in time for its recommendations to be considered for inclusion in the then President's budget for the fiscal year 1985–86.

Stephen Budiansky

Bad manners, USA

SIR — I recently had the pleasure of participating at the XVth International Congress of Genetics in New Delhi. I would like to congratulate my Indian colleagues for their successful effort. The congress was well organized, the atmosphere was friendly and congenial and the subjects covered were interesting and diverse. It was one of the best international congresses in the past few years.

However, I feel bound to apologize to the Indian organizers for the rude, inexcusable and unacceptable behaviour of a number of my American colleagues who never appeared at plenary sessions or symposia (as chairmen or as speakers). Their names were called without answer. Obviously their commitment to a "developing" country's meeting was not binding. Furthermore, quite a few failed to send a summary of the paper they were presenting at a symposium. Maybe they do not know that Indian students and young scientists are more eager to read than their counterparts in the United States.

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Israel squeezed out

SIR — Israeli scientists will not be able to participate in the 7th International Biotechnology Symposium to be held in New Delhi, India, 19–24 February 1984.

According to the organizers, scientists from countries without an Indian Mission will obtain entry permits/visas if the organizers are contacted at least six months before the symposium. Since July 1983, I have been trying to obtain information on how to apply for a visa, but to no avail. This symposium is sponsored by international organizations such as the International Committee on Economic and Applied Microbiology and the International Union of Pure and Applied Chemistry and should therefore be open to scientists from all countries of the world.

I call upon fellow scientists to boycott this meeting to show their solidarity and to protest to the organizers and the Indian Government that scientists from all countries cannot participate in the New Delhi meeting. The Indian Government's attitude should also be kept in mind when selecting sites for future international meetings.

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Creationist view

SIR — Professor Marsden overlooks some important points and makes some logical errors in his recent commentary on the history and sociology of the creation/evolution debate¹. In his search for a hidden agenda in creationism, he

overlooks the legitimate scientific case for creation, and he does not deal with possible philosophical, political, and economic motivations of the other group which seeks to polarize the debate — the evolutionary humanists².

He complains about the polarization of the issue as if a middle ground of progressive creationism, based on directed macro-evolution and an old Earth, is supported by scientific data. Professor Marsden has misunderstood radiometric dating and the geological time scale as simple scientific facts (SSFs), just like Newton's third law or the fact that a granite contains potash, feldspar and quartz.

If there were SSFs which indicated that the Earth is old and precluded *fiat* creation, then his point would be well taken. But the methods of evolutionary/uniformitarian geochronology, historical geology and palaeoenvironmental analysis are not SSFs, but rather complex scientific models (CSMs) involving many assumptions, some of which are demonstrably wrong, such as pleochroic haloes in granitic rocks, which are held to prove the constancy of radioactive decay rates through Earth history. Some of these data which argue against the doctrine of uniformity have even been published in *Nature*³. The historical assertions that the Cambrian began about 600 million years ago and that the Scopes trial began in 1925 are not equivalent concepts and, as such, should not be taught in the same breath to unwitting students.

Professor Marsden is also wrong in his thesis that we creationists are only a product of some "Post-Civil War cultural crisis". Creationists are convinced by coercive scientific data⁴, not solely by conservative theology, demography or profession.

As a fundamentalist, southerner and geologist, I resent several accusations made by Marsden and the implication that a conservative exegesis of *Genesis 9* can support slavery and that this is part of southern creationists' motivation. I also question his assertion that many southern anti-evolutionists are made because of "the relatively low levels of education of many Southern Bible-believers". I am a creationist because the basic facts of geology (and the other sciences) are consistent with *fiat* creation and several thousand years of Earth history dominated, during brief periods, by processes of catastrophic rate, on a continental scale and of high intensity⁵.

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Page charge problem

SIR — In scientific publishing a symbiotic equilibrium had been established in which referees evaluate, without payment, papers submitted for publication, in the knowledge that refereeing and publication of papers they themselves submit will be carried out without cost to them. Unfortunately, this equilibrium has been disturbed by a considerable number of (I think exclusively) US journals which now require the prospective author to pay for publication. Many people are therefore being asked to contribute to the publication of journals both by giving free service as referees and also by paying "page charges".

I understand that provision is made on many US research grants for the cost of publication. This is not so in Europe, and it is a practice which, in effect, discriminates against non-US scientists wishing to publish in US journals. Whilst the practice eases the problem for US scientists, the principle of working for, and paying to publish in, the journal seems wrong.

None of us expects or wants payment for refereeing manuscripts. The situation as it existed for many years (and still exists in Europe) was a mutual-help system to which we contributed and from which we benefited. But many scientists and their financiers are unhappy at the extra financial load imposed by some journals.

As a partial solution I propose that referees should ask for page-charge credits which could, wholly or in part, be used to offset the cost of their next publication. In this way no money would change hands and work done by the reviewer would simply result in the amelioration of an added financial burden upon research institutions. The best arrangement, of course, would be to return to the original equilibrium where we paid, and were paid, nothing.

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Sea cow born again?

SIR — If Steller's sea cow was first discovered in 1759 (as Vera Rich claims in *Nature* **306**, 415; 1983) how did the mining engineer, Retr Yakovlev know it was the "Kapustnik" and fear its extinction in 1755?

R. PARR
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● Vera Rich responds:
Rhytina stelleri
Became extinct early,
After brief fame begun
In 1741.
(1759 was a printer's error).

New twist for anthropic principle

The originator of the doctrine that our observations of the Universe are biased because they are made by us now concludes that there have been at most two critical steps in biological evolution.

THE anthropic principle is Dr Brandon Carter's choice of a philosophical middle ground between what he calls the "primitive anthropocentrism" of the pre-Copernicans and "its equally unjustifiable antithesis" of extreme representations of the relativistic cosmological principle, the assertion that, "apart from local inhomogeneities", no place (or time) in the Universe can be privileged in any way. When Carter introduced his principle a decade ago (*IAU Symp.* 63, 291; 1974), his objective was modestly to point out that the mere fact that our observations of the Universe are made by people who are the products of some 3,000 million years of biological evolution implies an inescapable bias. Carter's latest version of the argument, now published as part of the report of last year's meeting at the Royal Society on the constants of physics (*Phil. Trans. R. Soc. A* 310, 347-363; 1983) is more radical, and a challenge to biologists as well as astronomers.

The reasons why the anthropic principle should figure in a symposium on the constants of physics are not as surprising as they may seem. The luminosity of a star of given mass, and thus the speed of its evolution from a young star to, say, a red giant, depends linearly on the value of the gravitational constant. If this value were only a little greater than it is now thought to be, the Sun would have run through its evolution faster, so that biological evolution might have got no further than, say, the dinosaurs before the Earth was engulfed by a red giant. And a smaller gravitational constant might never have allowed for the particular circumstances that led to the emergence of living things in the first place. So there is a chance of learning something about the value of the gravitational constant simply by exploiting the circumstance that its evaluation depends on observations made by people. Carter now says that if he had known that the name "anthropic principle" would be so widely used, he would have looked harder for a different way of referring to the bias caused by self-selection.

In passing, Carter directs well-aimed darts at two fashionable preoccupations, speculation about extraterrestrial life and Dirac's large-numbers hypothesis, each of which he says is sustained by neglect of the anthropic principle. Telescoped, the argument is this. The search for extraterrestrial life is based on the hypothesis that the emergence of living things is likely

whenever geophysical circumstances are favourable, and thus on a denial of the contradictory hypothesis that the emergence of life is improbable. But, says Carter, the only evidence bearing on the question is the observation (by ourselves) that life has indeed emerged on the surface of the Earth, whence there is no way of distinguishing between the two hypotheses.

Dirac's large-numbers hypothesis is logically identical. Dirac at the outset argued that coincidence between the age of the Universe (strictly, the Hubble time) and the gravitational constant is so remarkable that one must expect the gravitational constant to decrease with time so as to preserve the coincidence. (Numerically, the Hubble time is 10^{10} years in units in which the velocity of light, Planck's constant and the gravitational coupling strength are determined by the three-halves power of the square of the proton mass, of the order of 10^{38} in the same units.) Carter's view is that the coincidence is not a coincidence at all, but a consequence of the selection bias occasioned because the observations are necessarily made by people whose existence requires a Hubble time large enough so that some stars can have completed their evolution and have generated the chemical elements of which people are made, but not so long that all stars have run their course.

This is the stuff of lunch-room arguments about the anthropic principle in the past decade. Carter's new departure begins with another coincidence — that between the time-span of biological evolution on the surface of the Earth (say, 0.4×10^{10} years) and the length of time the Sun is likely to spend as a hydrogen-burning star (say 10^{10} years) before becoming a red giant with dimensions engulfing the Earth's orbit.

What connection can there be between two such apparently independent quantities as the timescale of biological evolution, presumably determined by the complexity of living organisms, and the timescale of stellar evolution, determined (in Carter's words) by "the weakness of gravitation"? None, he says. The apparent coincidence is merely a consequence of the "habitual mistake" of overlooking the anthropic principle.

The most plausible way of accounting for it is to suppose that the timescale of biological evolution, itself a Markovian process (one thing leading to another) whose course must be uncertain, is on the average much longer than the time allowed

by the lifespan of the Sun as a hydrogen-burning star. So the observation, after a mere 4,000 million years, that there are sentient beings able to contemplate their own emergence is a sign that, in the event, the course of evolution on the Earth has been exceptionally short.

Carter takes this argument one argumentative step further. Suppose, he says, that the evolution of sentient beings depends on n critical steps (defined as unlikely evolutionary steps, which are thus "slow", compared with the majority of evolutionary steps). Then it is possible to show that the chance of all these n steps being completed by time t is proportional to t^n and also to show that evolution (the Sun's time as a hydrogen-burning star or T_0) exceeds that occupied by evolution so far by a quantity of the order of $(1/n)t$. But this time is itself of the order of T_0 , whence Carter concludes that n must be a whole number greater than zero and less than or equal to two — in short, either 1 or 2. Carter insists that the general character of this result does not depend on the details of the calculation but acknowledges that it poses a dilemma: how can the fossil record be squared with the conclusion that there have been at most two critical steps in evolution so far? His own preference is to face the dilemma head on; he recommends the evolution of the genetic code and the organization of the nervous system as candidates for the two critical steps.

What weight should be given to this conclusion? In his paper, Carter does not seek to mollify his critics, dismissing the attention being paid to the "inflationary universe" in all its varieties as an attempt to resurrect the "beguiling notion" of the perfect cosmological principle, and complaining that those who hold that only falsifiable hypotheses are admissible are in effect saying that science has accomplished nothing so far.

The Popper school will be further offended that Carter not merely uses the verb "to induce" but that he openly uses the language of Bayesian probability, the calculus of informed guesswork as some consider it. Even his friends may say that he has too lightly discarded the possibility that his coincidence may be explained if evolution is a rapid process which happens on the Earth to have been slow. But Carter is plainly not afraid to stir up trouble and has also provided a tangible example of how the anthropic principle may be made quantitative.

John Maddox

Ecology

A test of ideas about mutualism

from Robert M. May

THE past few years have seen some sharp debate over the extent to which competitive interactions are of importance in determining the structure of plant and animal communities^{1,2}. In this debate, it is sometimes claimed that the 1960s and 1970s were an era in which researchers regarded competition as essentially the only force moulding community structure. Such charges are clearly exaggerations, as these two decades equally saw an upsurge in empirical and theoretical work on predator-prey and parasitoid-host interactions. Any contemporary ecology text, American or British, reflects this attention given both to competitive and to predator-prey associations. What is true, however, is that mutualism has remained relatively neglected — in field, laboratory, theory and textbooks — until recently.

Why this relative lack of attention to mutualism? My view is that it is partly because tight mutualistic associations between clearly distinct species (as opposed, for example, to entities like lichens, where algae and fungi have fused into something akin to a single organism) are less conspicuous in temperate regions than are competitive or predator-prey associations; only relatively recently has there been much ecological research in the tropics where mutualistic interactions are conspicuous elements of community organization. The idea that obligate mutualisms may be dynamically fragile (and consequently more likely to be found in the environmentally stable tropics) essentially goes back to Darwin and Wallace, who both noted that obligate associations between plants and pollinators are not easily transported to islands. These ideas are explored in more detail elsewhere^{3,4}.

D.S. Wilson has advanced an interesting alternative explanation for this relative neglect of mutualism⁵. He argues that the controversies about group selection in the 1960s led to a sharper recognition — crystallized in Williams's important book⁶ on *Adaptation and Natural Selection* (1966) — that conventional Darwinian selection acts on individual organisms, not on groups or species as such. Much of the earlier discussion of mutualism, however, made implicit or explicit appeal to adaptations that benefited groups rather than individuals. Wilson notes that many older textbooks, such as the influential *Principles of Animal Ecology* (1949) by Allee *et al.*⁷, had treated mutualism as a dominant interaction in well-ordered communities; but the explanation was in terms of the adaptation of species rather than individuals. Williams argued that mutualism can evolve, but only if conditions are such that it is advantageous for the individual in-

involved, and he went on to observe⁶ (p.247) "really good examples of mutualism are relatively rare, and it must be that these necessary [conditions] seldom arise".

Contemporary theory, as developed by Wilson^{8,9} and Wade¹⁰ (and reviewed by Uyenoyama and Feldman¹¹ and others¹²), considers more explicitly the effects that group size may have on the evolution of mutualistic associations. Suppose a species contains both mutualistic and nonmutualistic types: the mutualist benefits another species in its vicinity, but at a cost, c , to itself; the enhanced presence of the other species reciprocally confers a benefit, b , on all N individuals (mutualists and non-mutualists alike) in the group of which the mutualist is a member. It is clear that the proportion of mutualists must decrease in any group containing both types, because they alone bear a cost while the entire group benefits: this constitutes an evolutionary force against mutualism. But groups with many mutualists will, by virtue of the benefits conferred, produce more offspring than those with few mutualists: this constitutes an evolutionary force favouring mutualism. Thus these models suggest that⁵ "the evolution of mutualism is a balance between the opposing forces of relative fitness within groups (individual selection) and the differential productivity of groups (group selection)". The outcome will depend on the costs c , benefits b and — most important — on the degree of variability in group sizes (which variability will, under most statistical assumptions, be more pronounced if the average group size N is relatively small): mutualism will be favoured by small c , large b and small N .

Unfortunately, experimental tests of these ideas are difficult. For one thing, the parameters c and b can be hard to define, much less measure, in field settings. For another, the effective group size N may be smaller than it appears, either because apparently large groups actually derive from a small number of colonists, or because the population structure results in very large variation in size among groups (promoting the evolution of mutualism).

Wilson has fastened on the association between burying beetles and the mites they carry around as a system that may be particularly amenable to field and laboratory exploration of these questions about mutualism⁵. The associations he has been studying include five sympatric species of burying beetles of the genus *Nicrophorus*. These creatures are carrion feeders. They raise their broods on small carcasses (mainly dead rodents); the carcass is first buried, and the beetles then remove its hair and roll it into a ball. This remarkable sequence of actions is carried out by a pair of beetles,

after which the male leaves while the female remains until her brood has completed its larval development. In northern Michigan, carried around on these beetles are some 15 species of mites from four families and at least one species of nematode. These 'phoretic' mites disperse to new habitats by attaching to the beetles and then abandoning their carriers at the new habitat to follow a free-living existence; the larval mites develop on buried carcasses, side by side with the beetle larvae that represent the mites' next 'aeroplane'. When mites leave their beetle host to take up residence on a buried carcass, they search out and destroy (by piercing with their chelicerae) fly eggs that may be present; thus the mites may benefit the beetles by reducing competition for carcasses by flies. Finally, Wilson observes that mixing of adult mite populations takes place at large unburyable carcasses where large numbers of beetles congregate to feed.

In short, these associations between phoretic mites and burying beetles exhibit the conjunction of characteristics envisioned in the models described above: on the one hand, local 'trait groups' of different sizes, in which mutualistic effects may be present, are found on individual buried carcasses; on the other hand, there is mixing of groups at the large carcasses where adult beetles feed communally.

Wilson focuses mainly on field and laboratory studies of the association between the mite *Poecilochirus necrophori* and the beetle *N. tomentosus*. He shows that such beetles carry on average about 12 *P. necrophori*, which (given that carcasses are buried by a male and female pair of beetles) makes the average group size rather large, $N \sim 24$; Wilson also shows there is much variability in the size of these groups. A complicated series of experiments can be summarized as broadly leading to the conclusion that the mites' habit of piercing the eggs of flies strongly enhances the breeding success of the carrier beetle species: in field studies, *N. tomentosus* raised broods of average weight 0.092 g from 26 of 30 buried carcasses when *P. necrophori* was present, while broods of average weight 0.088 g were raised from 15 of 28 buried carcasses in the absence of *P. necrophori*; this overall twofold difference in reproductive success is statistically highly significant.

Wilson discusses the possibility that the mites may pierce eggs purely to feed on them, so that the beneficial effects to the beetles are incidental rather than the outcome of a co-evolved mutualism. Were this the case, it would be expected that mite fecundity would be correlated, to some extent, with the presence of fly eggs. But a variety of laboratory and field experiments shows no correlation between mite fecundity and abundance of fly eggs; even the total absence of eggs has no discernible effect, as the mites reproduce well on the carcass itself. Costs associated with the egg-piercing behaviour are harder to document

Last gape of the Tasmanian tiger



THAT most curious of marsupials, the thylacine or 'Tasmanian tiger', is commonly assumed by zoologists to be extinct. The last known specimen died in captivity in Beaumaris Zoo, Hobart on 7 September 1936 after a deadly campaign led by Tasmanian sheep-farmers over the preceding half-century. Anecdotal reports of more recent encounters notwithstanding, no solid evidence has been forthcoming since then despite several major expeditions and many forays by wildlife enthusiasts.

Thylacinus cynocephalus — literally 'pouched dog with a wolf head' — managed to combine a lupine visage with a tiger's stripes, a snake's gape, a kangaroo's

tail and a unique rear-opening pouch (the thylacine loped about on all fours and would otherwise have risked injury to its young from rough undergrowth). From nose to tip of tapering tail the thylacine measured up to 2 metres, in height 55 cm and in weight 20 kg, thus qualifying as the largest carnivorous marsupial. Although its tawny fur and other external characteristics can be seen in pelts preserved at the British Museum (Natural History) and elsewhere, the astonishing capacity to open its jaws to an angle of over 120° is best appreciated on this unique surviving photograph, the last recorded gape of the Hobart specimen *circa* 1935. The two frames are reproduced from an 8-mm film

by courtesy of the State Library of Tasmania.

The latest in a long line of claimed sightings was made in 1982 in northwestern Tasmania by a ranger of the National Parks and Wildlife Service, according to just released press reports. The news had been kept secret to deter hunters and sight-seers, but the deployment of trip cameras and sand traps over the last eighteen months yielded neither photographs nor spoor. □

This is the first in a series of historic scientific photographs which will be appearing in News and Views. They are selected from Beyond Vision by Jon Darius, to be published in April by Oxford University Press.

(and if there are no costs, then in the above models mutualism can of course evolve in groups of arbitrary size). Wilson suggests two kinds of cost, which he hopes to quantify in future experiments. First, since the mites' diligence in piercing every egg they can find apparently pays no dividend in enhancing reproductive success, it represents a departure from optimal foraging; this is likely to carry some cost, even in the mites' food-rich environment. Second, the habit of *P. necrophori* of leaving its beetle carrier upon discovery of a carcass incurs the substantial risk of the carcass being appropriated by some other species of animal. As Wilson observes⁵, "A mite that abandons its carrier only to have it fly away has made a poor choice, and I do occasionally observe a few *P. necrophori* wandering forlornly around a half-built burial chamber that has been robbed by a mammal. Yet the carrier must be abandoned to pierce fly eggs. Assuming that the beetle does not force abandonment, this may represent the greatest potential cost of the mutualistic behavior".

As Wilson emphasizes⁵, this research programme is still in its formative stages.

The existing work does a lot towards quantifying the benefits and the population structure of one particular mite-beetle association, but there is a need for more information (particularly about the costs). Beyond this, it can more generally be remarked that burying beetles live in a very competitive environment, where their relatively slowly developing larvae (the eggs hatch several days after the carcass is buried, and larvae take 10 to 20 days to complete their development) must contend both with faster developing flies (where the span from egg to adult fly takes seven days or less) and with the problem of 'spoilage' of the carcass by microbial activities. The mite *P. necrophori* is just one member of a diverse phoretic community; *N. orbicollis* species in northern Michigan, for example, typically carry seven species of mites and at least one nematode species. Wilson suggests that, as most of the mites filter micro-organisms, these mite-beetle associations may represent elaborately co-evolved mutualisms (with prevention of spoilage of the buried carcass being an important component), in spite of average group sizes exceeding 100 mites per beetle. He concludes

that⁵ "it seems possible that the beetles and their phoretic associates have congealed into a multispecies mutualistic network that systematically alters the local environment in ways that cause its own perpetuation. If so, then the structure of these miniature communities more closely resembles the view of Allee *et al.*⁷ than more modern ecological theories". In its larger aspects, Wilson's experimental study will of necessity unfold slowly. □

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Gene expression

The end of the message and beyond

from Nick Proudfoot

SINCE last reviewed in these columns a little over a year ago¹, several major steps forward have been made in understanding how the 3' ends of eukaryotic mRNA are formed. First and foremost, histone genes have joined other eukaryotic RNA polymerase II genes in generating their 3' ends not by simple termination of transcription, but by termination followed by post-transcriptional cleavage. The activity responsible for this process has been nearly purified and looks very much like a small nuclear riboprotein or 'snurp', not the U1 snurp responsible for intron splicing, but another as yet unidentified one. Second, mutation of the AAUAAA conserved sequence found at the 3' ends of poly(A) containing mRNAs has been shown to cause elongation of the mRNA transcript². This formally proves that the AAUAAA sequence is required for mRNA 3' end formation, and strongly suggests that the sequence is a signal for endonucleolytic cleavage. Finally, the feeling that RNA polymerase II must have a precise termination process, rather than just falling off the gene template at random, has been virtually proven, at least for the mouse β major globin gene.

Max Birnstiel's laboratory in Zurich has been working on the transcription of sea urchin histone genes by injecting them, together or separately, into *Xenopus* oocytes^{3,4}. Not surprisingly, several of the activities necessary for the generation of sea urchin histone mRNA are absent in oocytes. Birnstiel's group therefore tried co-injecting sea urchin extracts along with the histone genes to complement the oocyte transcriptional apparatus⁵ and found that with the extracts, histone gene H3 does generate mRNA that terminates at the correct 3' position, while without the extracts it does not. Purification of the activity using this complementation factor assay⁶ has now revealed that it is a nuclear RNA-protein complex known affectionately as a 'snurp'. Indeed, even a 60-nucleotide RNA fraction free of protein stimulates the production of correct histone mRNA 3' ends when injected along with the histone gene into oocytes — presumably oocytes can package the RNA into a snurp⁷. Although this group's experiments strongly imply that histone mRNA 3' ends are generated by endonucleolytic cleavage, the final proof has come from other work.

Krieg and Melton of Harvard University⁸ took a different histone gene, placed it under the influence of a bacteriophage (SP6) promoter⁹, and synthesized *in vitro* transcripts extending beyond the 3'

end of the mRNA. When these RNAs were injected into oocytes, correct histone mRNA 3' ends were created, proving that a precise RNA endonuclease activity is present in oocytes, capable of producing histone mRNA 3' ends. Similar results have been obtained by Birnstiel's group (unpublished data) and by Carl Parker at Caltech (personal communication). Presumably the 'histone snurp', either single-handed or with the help of other activities, cleaves the histone mRNA primary transcript at the mRNA 3' end position.

Birnstiel's group has now gone on to define rather precisely what these activities recognize in the primary RNA transcript¹⁰. Clearly, the hairpin at the histone mRNA 3' end is all-important, although site-directed mutagenesis shows it does not matter exactly what sequence the hairpin has, as long as base-pairing is maximized. Also, the immediate 3' flanking sequence to the hairpin structure appears to be necessary to form the mRNA 3' end. In other words, the recognition site for cleavage is fairly complex — and that will make the precise biochemistry of the RNA-protein interactions hard to unravel.

Understanding of the processes involved in the formation of poly(A)-containing mRNA 3' ends has similarly progressed. The conserved sequence AAUAAA is clearly important as two site-directed mutagenesis experiments show. In one case¹¹, the adenovirus E1A poly(A) site was mutated to AAGAAA, causing all E1A transcripts to extend into the next E1B gene as cleavage at the E1A mRNA 3' end was prevented. In the second case¹², experiments on a new type of non-deletion α -thalassaemia discovered in Saudi Arabia (Saudi α -thal) have shown that the human $\alpha 2$ gene contains a single base change in the poly(A) site, AAUAAG, that again results in the production of α -globin mRNA with 3' ends extending way beyond the normal 3' end. These transcripts are unstable in human reticulocytes so insufficient globin is produced and the α -thalassaemia phenotype is seen.

Clearly, these two studies strongly suggest that AAUAAA forms part of a recognition signal for the endonucleolytic cleavage necessary to generate a correct mRNA 3' end. A recent theoretical study by Susan Berget¹³ neatly lends support to this presumption. She argues that the AAUAAA sequence, together with another less convincing sequence homology CappyUG, usually found close to the site of poly(A) addition¹⁴, can base-pair reasonably well to a small nuclear RNA called U4. She assumes, of course,

that cleavage at the AAUAAA sequence is yet another snurp-mediated process.

Considerable progress has also been made in understanding the first snurp to be talked about, U1. Since the original sequence homology arguments^{15,16} that suggested U1 could base-pair with intron donor and acceptor sites^{17,18} and thereby facilitate intron splicing, several more direct experiments have been carried out. Work in Joan Steitz's laboratory, where the term 'snurp' was first coined¹⁹, has demonstrated the direct interaction of U1 with an intron donor site²⁰. Also, very recently, *in vitro* splicing of adenovirus introns has been convincingly demonstrated in HeLa cell extracts^{21,22}. Using this *in vitro* splicing system, Phillip Sharp's laboratory, in collaboration with Joan Steitz, has demonstrated the inhibition of *in vitro* splicing by antibodies directed against U1 snurps²³. Further evidence comes from Hernandez and Keller²² who have partially purified one 'splicing' component of the *in vitro* splicing extract and shown that this fraction co-purifies with snurps.

Finally, yeast introns have been shown to contain their own U1-like sequence actually as part of the intron sequence close to the 3' acceptor site^{24,25}. If it performs the right contortions the sequence may be able to interact with the donor site and mediate intron excision. It would thus seem that there is one general mechanism for the post-transcriptional processing of RNA polymerase II gene transcripts: each cleavage event, be it the removal of an intron or the formation of a mRNA 3' end, is initiated by specific sequence recognition between the gene transcript and a small nuclear RNA. Activities associated with this RNA (that is, the snurp proteins) then cleave the gene transcript and are followed by RNA ligation in the case of intron removal, or by polyadenylation in the case of mRNA 3' end formation.

Returning to the 3' end of the mRNA, if, as described above, both histone and poly(A)-containing mRNAs generate their 3' ends by RNA cleavage, then where does RNA transcription actually stop? Specific RNA termination sites have been demonstrated in bacteria, so we might expect eukaryotes to have them too — and indeed this has now been shown to be the case, at least for the mouse β major globin gene. Jim Darnell and his colleagues at the Rockefeller University, New York, have precisely pinpointed a position about 1 kilobase beyond the end of the gene, beyond which no RNA transcripts can be detected^{26,27}. They used a tissue culture cell line — the mouse erythroleukaemic Friend cell — that can be induced by various chemicals such as dimethyl sulphoxide to express high levels of globin mRNA and protein. By pulse-labelling the cells with [α -³²P]UTP, newly synthesized precursor RNAs were ³²P-labelled. Nuclear RNA was then purified and hybridized to single-strand 3' flanking region DNA fragments

cloned into the single-strand bacteriophage vector M13. The RNA-DNA hybrids obtained were next treated with ribonuclease and the protected RNAs fractionated on gels. One 3' flanking region M13 clone protected RNA for only half of its length. The 3' flanking region M13 clones taken from in front of this clone protected RNAs for their whole length, whilst the 3' flanking region M13 clones from beyond the clone produced no protected RNA. These results clearly show the presence of a discrete RNA 3' end well beyond the end of the mRNA. It seems likely that Darnell's experiments have identified a eukaryotic termination site. If such a site exists in the mouse β major gene, then it seems plausible that many, if not all, eukaryotic genes have termination sites.

Why are the termination of transcription and the formation of mRNA 3' ends separate processes? Well, what at first sight appears an unnecessary complexity may perhaps give an additional control over gene transcription. We may find that specific factors modulate the efficiency of transcriptional termination. If termination were prevented, then either the primary transcript could be rendered even more unstable, so that less mature mRNA could be produced from it before it turned over, or alternatively, transcription might read through into the next gene and disrupt its normal transcription. Obviously, these possibilities are only speculation. But the view that all the controls that modulate eukaryotic gene expression centre on the promoter may well prove too narrow. □

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Developmental biology

Morphogenesis by fibroblast traction

from Julian Lewis

THE fibroblasts that make up the extracellular matrix in the adult body may seem dull, commonplace cells, cast in a supporting role while others play the star parts. But fibroblasts in the embryo appear in a more interesting light. They are the cells that carry much of the information that dictates the structure of the skeleton¹, the locations of the muscles², the routes of the nerves³ and the patterning of the skin⁴. They are the architects of connective tissue as well as the makers of the raw materials. They work on the collagen that they have synthesized and secreted, compacting it into sheets, drawing it out into cables, crawling over it, tugging upon it; and with its help, controlling the disposition of other cells and organs⁵.

Though fragments of evidence have been available for a long time⁶, it is only recently that experiments have brought the mechanical role of embryonic fibroblasts clearly into focus. A fibroblast alone in a culture dish will crawl about: it extends its leading edge, adheres with it to the substratum and uses the new site of anchorage to pull itself forwards, probably by an actin-based contractile mechanism. In the process, the fibroblast elongates itself for its trailing edge also is stuck to the substratum and does not easily detach.

Since action and reaction are equal and opposite, a force must be exerted on the substratum, tending to put it into compression in the region between the two ends of the fibroblast and into tension beyond them. The force can be made visible by culturing the cell on a very thin sheet of silicone rubber, which is thrown into compression wrinkles beneath the cell and into tension wrinkles beyond its ends⁷. The compression wrinkles run transverse to the long axis of the cell, while the tension wrinkles radiate from its ends.

Fibroblasts show their strength in a more dramatic way when they have collagen fibres to work on, instead of a rubber sheet. The clearest demonstration has come from experiments using an artificial collagen gel prepared from a solution of dissociated collagen subunits which is poured into a Petri dish and allowed to set by re-association, forming a mesh of randomly oriented collagen fibrils. Living fibroblasts can be added so as to create a sort of artificial loose connective tissue in which the cells are uniformly dispersed. The gel then contracts away from the walls of the Petri dish, squeezing fluid out of the meshes of the collagen, and by the end of the week is compacted into a pellet with a diameter which may be less than a fifth of the original — corresponding to a volume less

than a hundredth of the original^{5,8}. The contraction occurs only if fibroblasts are present, and it can be prevented by adding cytochalasin, which is known to disrupt actin filaments and hence the intracellular contractile mechanisms that depend on them⁸. If vertical pegs are fixed to the base of the dish to prevent contraction of the gel, then collagen is stretched into rope-like bands that loop around the peg and are pulled so taut that they will often snap⁵.

The contraction of the artificial gel is clearly similar to the contraction of granulation tissue in wound healing, well known to pathologists, who have coined the term 'myofibroblast' to describe the muscle-like nature of the fibroblasts that bring it about⁹. In fact, studies of wound contraction provided the earliest clear evidence of the force-generating role of fibroblasts in a collagen matrix⁶.

There is, however, a major difference between the action of fibroblasts and that of muscle cells. Muscle cells are permanently attached at both ends to other structures, and bring about contraction of the tissue simply by shortening themselves. The fibroblast does more: while exerting a contractile force, it changes its points of attachment, hauling in more and more collagen, as a sailor hauls rope hand-over-hand. Indeed, the analogy goes further. Just as sailors use their ropes to manoeuvre the masts and sails, so fibroblasts use collagen fibres to pull the parts of the developing body into their proper positions; and just as sailors can swarm up the rigging, so fibroblasts can swarm along the collagen fibres that they have themselves formed and aligned.

These points are illustrated by further experiments *in vitro*⁵. If a small chunk of tissue containing fibroblasts is excised from a chick embryo and placed in a collagen gel that is otherwise cell-free, it will begin to draw in collagen from its surroundings, creating for itself a capsule of densely packed and circumferentially oriented fibres, and further out, beyond the most peripheral fibroblasts, setting up stresses that orient the collagen radially. If two such explants are placed far apart in the gel, a very striking 'two-centre effect' is seen: along the line that joins them, in the cell-free region, the collagen becomes organized into a compact band of aligned fibres¹⁰. Thus if embryonic cartilage rudiments, stripped of loose connective tissue but retaining a thin layer of perichondrial fibroblasts, are placed in the gel, they recruit for themselves thick new coats of perichondrial collagen and become linked by oriented collagen bands resembling

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ligaments.

If small chunks of embryonic muscle tissue (consisting of muscle cells mingled with fibroblasts) are also placed in the dish, they become connected to the cartilage rudiments and to one another by bands of collagen equivalent to tendons; the muscle fragments, initially shapeless blobs in random positions, become stretched out into well-formed muscle bellies and are towed into place by the collagenous attachments to the skeleton⁵. Paradoxically, the muscle cells appear to be the passengers rather than the engines of such movements. In an embryonic limb bud that has been deprived of cells capable of developing into muscle, the skeleton at first forms normally, and so do the tendons, even though they have no muscles to pull on them¹¹.

Oriented fibrils in an extracellular matrix can orient cell movements (as a paper in this issue of *Nature*, p.453, from Norio Nakatsuji and K. Johnson demonstrates in the context of the amphibian gastrula¹²). When fragments of tissue are explanted into a collagen gel, cells — presumably fibroblasts — migrate out into the uninhabited regions of the gel; and the migrants move most rapidly, and in largest numbers, along the dense oriented bands of collagen that have been created by fibroblast traction. Thus the fibroblasts govern the disposition of collagen, and the collagen in turn affects the disposition of the fibroblasts.

Furthermore, a strong and active cluster of fibroblasts surrounded by collagenous matrix will tug towards itself not only the matrix but also any fibroblasts that may be clinging to it. Once recruited into the cluster, these cells will add to its strength. Thus inhomogeneities in the distribution of fibroblasts will tend to be self-amplifying.

Clearly, fibroblasts and collagen fibres together make a most remarkable dynamic system. A recent paper defines its properties in mathematical terms and discusses its capabilities for pattern formation¹³. For example, a homogeneous

tissue of fibroblasts dispersed in a random matrix may become unstable against small departures from uniformity, and so spontaneously develop a pattern in which the cells become clustered and the collagen becomes organized into tracts.

Just such a phenomenon is seen in the skin of a bird embryo as feather tracts form. The fibroblasts of the previously almost uniform embryonic dermis gather into a regular lattice of condensations, corresponding to feather rudiments, linked by oriented bands of collagen. The whole transformation is blocked in the presence of collagenase¹⁴. In appropriate conditions, an artificial gel of collagen containing dispersed fibroblasts will show roughly similar behaviour^{15,16}. Theory and experiment here converge in a most persuasive way.

A bolder and more dubious application of the theory of fibroblast traction aims to explain the origins of the pattern of long

bones in a developing limb as a result of mechanical instabilities in the mesenchyme of the undifferentiated limb bud, creating non-uniformities where none existed before¹³. Limb-bud specialists are likely to be sceptical: there is too much evidence that cells of the apparently undifferentiated mesenchyme are already intrinsically different and determined for the formation of specific skeletal structures even before any cartilage 'condensations' are visible¹; and the patterning of the skeleton seems too insensitive to artificial and mechanical disturbances. There is surely more to pattern formation in the developing limb than mechanical instabilities generated by fibroblast traction: but it is no less sure that fibroblast traction plays a major part in the process. □

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Obituary

Ahmad Bukhari 1943–1983

from Neville Symonds

AHMAD BUKHARI had exercised a formative influence on research in transposable elements for close to a decade and was at the height of his powers when he died suddenly of a heart attack on 19 November 1983.

His earliest major contribution, in 1975, concerned the excision of transposable elements, an issue he always felt was undervalued in the rush to understand how replicative transposition occurs. He was able to show that most bacteriophage Mu insertions in a host gene could be excised exactly with the restoration of gene activity, so demonstrating for the first time that Mu transposition normally occurs without any damage to target DNA sequences.

With regard to replicative transposition, he and Ljungquist published a crucial paper in 1977 showing that coupled transposition and replication of Mu proceeds without Mu leaving its original prophage site. Using an ingenious combination of restriction analysis and DNA–DNA hybridization, they examined the state of prophage Mu DNA after induction and were able to demonstrate conclusively that the original Mu–host junction fragments remained intact well into the latent period after many rounds of replication had occurred.

Bukhari's strength as a scientist rested on his ability to see problems as a whole and to appreciate the elements that were crucial to their understanding. This is exemplified in the beautiful review he wrote for *Annual Review of Genetics* (10, 389) in 1976, 'Bacteriophage Mu as a transposition element', in which he brought together the known experimental findings about Mu, assessed their importance from the view-

point of transposition, indicated the areas where further ideas and investigations were needed and finally related Mu behaviour to that of other insertion elements.

Recently, research on Mu at Cold Spring Harbor has focused on the mechanism by which Mu transposes, paying particular attention to the findings that transposition sometimes leads to replicon-fusions (alternatively called co-integrates) and sometimes to simple insertions. These studies led Harshey and Bukhari to propose a variant to the Shapiro model for transposition which has stimulated a considerable amount of theoretical discussion and practical activity, and which is supported by observation of Mu transposition intermediates using electron microscopy.

Bukhari was born in the Punjab and was an undergraduate at the University of Karachi, leaving for the US in 1966 to carry out graduate studies at Brown University and the University of Colorado. From 1970 he worked continuously at the Cold Spring Harbor Laboratory, and was the centre of one of the most active research groups working with bacteriophage Mu.

As a person Bukhari was emotional and enthusiastic with a variety of interests and friends. He maintained his connections with Pakistan, at the personal as well as the scientific level, and was a scientific adviser to both UNESCO and UNIDO. He will be sorely missed both for his intuition as a scientist and for his humanity as a friend. □

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Molecular biology

Multiple forms of mitochondrial DNA in higher plants

from Thomas D. Fox

THE study of the structure of the mitochondrial genomes of higher plants is a daunting task. Simply in terms of size there are problems enough — the genomes can contain from about 200 kilobases (kb) to over 2,000 kb of primarily unique sequence, according to species. In comparison, human mitochondrial DNA, which appears to code for roughly the same number of products, is only 16.5 kb long. Worse yet, electron microscopy of plant mitochondrial DNA reveals a complex assortment of differently sized circular and linear molecules, and restriction digests contain fragments in sub-stoichiometric amounts, suggesting a heterogeneous structure (for a review see ref. 1).

Some semblance of order in this situation has now been provided by the restriction mapping studies of Palmer and Shields², reported in this issue of *Nature* (see p. 437). Their results suggest that plant mitochondrial genomes are carried on discrete circular molecules which are derived from each other by recombination.

Chromosome walks along the mitochondrial DNA of *Brassica campestris* (Chinese cabbage, turnip) led around three differently sized circles. The largest, 218 kb long, contains the entire sequence complexity of the *Brassica* mitochondrial genome. This 'master chromosome' contains a major direct repeat of about 2 kb, whose elements are separated by 135 kb on one side and 83 kb on the other. The other two circles are 135 and 83 kb in size and each contains the corresponding sequences present between the repeats of the master chromosome and a single copy of the repeated element. (Other arrangements, such as dimers of the 218 kb circle, are consistent with the mapping data *per se* but fail to explain the stoichiometries observed for fragments containing the repeats and surrounding unique DNA.) Clearly, a simple way to account for these

species is to assume that they are related to each other by homologous recombination between repeated elements.

As Palmer and Shields point out, this relatively simple tripartite picture may turn out upon further study to be complicated by the presence of other minor species. One would, for example, expect the presence of dimers of the 83 kb and 135 kb circles to be present. Furthermore, Palmer and Shields detected, but did not map, other minor repeated sequences in *Brassica* mitochondrial DNA which may also serve as sites for recombination.

Mapping studies on the much larger mitochondrial genome of maize by Lonsdale and co-workers at the Plant Breeding Institute and University of Cambridge (personal communication) have indeed revealed a similar but more complex picture. They have mapped a maize mitochondrial DNA master chromosome of 570 kb and located six pairs of repeated elements within it: five direct repeats and one inverted repeat. In addition, six other smaller circular maps, ranging in size from 47 to 503 kb, were generated and could be derived from the master chromosome by recombination between pairs of repeated elements. These smaller circular maps seem to correspond well in size to circles identified in maize mitochondrial DNA by electron microscopy³. Thus the presence of multiple circular species of DNA, related by recombination between repeated elements, may be a general feature of higher-plant mitochondrial genomes.

The study of recombination in plant mitochondrial DNA has been hampered both by the lack of genetic markers and because mitochondrial DNA is maternally inherited. Nevertheless, evidence for recombination has been obtained by somatic cell hybridization of strains differing in polymorphic mitochondrial DNA restriction fragments⁴⁻⁶. The hybrids typically contain new combinations of parental fragment types, as might be expected from the assortment of multiple circular DNAs during mitotic growth. In addition, many hybrids contain novel fragments not found in either parent, which most probably arise as a result of true crossing-over. Complete restriction maps of plant mitochondrial DNAs will allow the detailed analysis of recombination in somatic cell hybrids.

Whether plant mitochondria contain a general recombination system, such as that present in yeast mitochondria, is not yet clear. They may contain site-specific recombination systems which only

function at some repeated elements — a possibility suggested by the finding of repeated elements in maize mitochondria DNA that are not surrounded by the expected recombinant set of restriction fragments (D.M. Lonsdale, personal communication). Site-specific recombination would tend to limit the number of different circles that could be generated from a master chromosome containing many repeats. (The large number of linear molecules in preparations of mitochondrial DNA from plants, as well as yeast, are assumed by most workers to be the result of *in vitro* degradation. Perhaps some of these linear molecules are recombination intermediates present *in vivo*.)

Progress in identifying plant mitochondrial genes through their homology with genes of other organisms will soon make it possible to see whether the arrangement of genes is conserved, as in animals, or not, as in fungi. Using the restriction maps of the maize genome, D.M. Lonsdale and C.J. Leaver of the University of Edinburgh (personal communication) have already shown that the genes coding for cytochrome oxidase subunit I, apocytochrome *b* and a protein homologous to that encoded by the human unassigned reading frame *URF1* are present in a cluster. The cluster also contains sequences derived from chloroplast DNA containing what appear to be pseudogenes for chloroplast 16S rRNA⁷ and the large subunit of ribulose biphosphate carboxylase⁸. About 200 kb away is another gene, for cytochrome oxidase subunit II⁹, and midway between them, the rRNA genes.

Comparisons between species such as *Brassica* and maize will also help give some clues as to why the majority of plant mitochondrial DNA sequences appear not to code for useful products. That chloroplast DNA sequences^{7,8} are found in maize mitochondrial DNA led Lonsdale to suggest that the ability to take up foreign DNA could in part account for the large and variable sizes of plant mitochondrial genomes. It will be interesting to see how true this is of other species, and whether *bona fide* nuclear DNA sequences also turn up in plant mitochondria. □

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100 years ago

BULLETIN No. 3 of the Entomological Division of the U.S. Department of Agriculture (Washington, 1883), when stripped of the "red-tape" that appears to be even more necessary on official documents in the States than it is in this country, is of more than usual interest. The notorious "army-worm" appears in a new character, viz, as destructive to cranberries, which form an important feature in the production of the States. A long chapter is devoted to the "cotton-worm" in which (in addition to interesting biological information) elaborate contrivances for distributing arsenical solutions are described.

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Geophysics

Palaeobathymetry from sinking shells

from Peter J. Smith

THE variety of phenomena from which information can be wrung about the geophysical past is a constant surprise. Consider, for example, the case of the sinking cephalopods.

A few years ago, J.S. Weaver and J.A. Chamberlain, building on earlier ideas and making use of the equations derived by naval architects to describe the sinking of ships, managed to work out the physics of the sinking of coiled and chambered shells (see, for example, *Math. Geol.* **10**, 673; 1978). They were able in particular to demonstrate, both theoretically and by laboratory experiment, that the depth to which an intact coiled cephalopod will sink with its plane of symmetry remaining vertical is governed only by the geometry of the shell. What was not clear at the time was whether, given the more chaotic conditions of the natural environment, observations of *in situ* vertical cephalopod shells could be interpreted sensibly in terms of palaeobathymetry. R.E. Crick has now shown that they can be (*Bull. Geol. Soc. Am.* **94**, 1109; 1983).

This is possible because of the remarkable series of events that overtakes chambered cephalopods after death. Gases released during the decay of the visceral mass often increase the buoyancy of a dead cephalopod so that the shell rises to the water surface where, unless it is stranded on a shoreline, it becomes waterlogged and sinks. But it doesn't just drop. Initially, the shell floats with its plane of symmetry vertical; but as it gradually absorbs water and sinks (and assuming it remains undamaged) it begins to rock from side to side until it finally becomes horizontal. The depths at which these changes occur depend on the shell's geometry. If the water is shallower than the rocking depth for a particular type of cephalopod, the shell will reach the bottom and become anchored, infilled and buried in a vertical position. Therefore, any ancient cephalopod shell discovered in a vertical position must have been deposited in water whose maximum depth can be estimated from the shell's geometry.

Of course, there are potential difficulties. For example, could a sinking shell, having reached the depth at which it becomes horizontal, nevertheless end up in a vertical position through impact at the bottom? That seems unlikely, for cephalopod shells float down much too gently to undergo a disorienting impact.

Second, could shells reaching the bottom in a horizontal state subsequently be turned to the vertical by bioturbation? They could indeed, although there are ways of detecting that in particular cases.

Third, it is necessary to assume

(reasonably) that gravity, seawater density and atmospheric pressure have not changed since the shells sank.

Finally, if the method is to work, it is essential that not all shells that end up in a vertical position at the bottom should subsequently topple over; but as vertical shells are actually observed, this appears not to be too much of a problem.

Crick has applied the technique to the top of the Fort Worth limestone, which extends in a narrow belt southwards from the Texas–Oklahoma border and which formed during the existence of the East Texas Embayment (Lower Cretaceous). There are enough vertical cephalopods (ammonites and nautiloids) to provide data at points throughout the whole length of the formation; and there were several different types of cephalopod present, so that maximum water depths can be determined over the range 1.6–2.6 m. Moreover, at the few points at which there are no vertical shells and hence at which it is not possible to specify maximum water depths, it has at least been possible to

estimate minimum depths, for the depths must have exceeded those at which the particular types of cephalopod begin to deviate from vertical sinking.

The conclusions that Crick reaches are that the Fort Worth cephalopods were laid down in water depths of generally less than 2.6 m and that depths increased slightly to the east and south. In the context of local geology, this is a considerable advance in that previous estimates of water depth based on less reliable (sedimentological and palaeoecological) criteria lie in the range 15–40 m. More important than the specific results, however, is that the method appears to work and is evidently of wider applicability. The only condition is that the formation to be studied should contain cephalopods laid down in water of a depth not exceeding the depths at which the various types of shell begin to depart from vertically oriented fall. □

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Astronomy

IRAS circular 7

The source name consists of four parts: (1) the letters 'IRAS' to indicate the origin; (2) the right ascension (RA) in hours and minutes, seconds omitted; (3) declination (Dec) in decimal degrees, multiplied by 10 and then truncated (thus $+32^{\circ}42.3$ becomes $+327$); (4) an appendix starting with 'P' and followed by the number of the circular; this appendix stresses that the data are preliminary. Position is given at Equinox 1950.0. The measurements were made between epochs 1983.1 and 1983.6.

Source IRAS	RA (1950)			Dec (1950)		Flux density (Jy)			
	h	min	s	deg	arc min	12 μ m	25 μ m	60 μ m	100 μ m
0000+818P07	00	00	12	+81	45.9	<0.2	<0.2	0.6	<1.6
0007+821P07	00	07	33	+82	08.4	<0.2	<0.2	0.6	<1.6
0119+868P07	01	19	26	+86	49.5	<0.2	<0.2	0.6	<1.5
0147+891P07	01	47	23	+89	06.7	<0.2	<0.2	0.8	1.9
0354+226P07	03	54	54	+22	33.8	<0.2	<0.2	<0.5	3.5
0358+194P07	03	58	04	+19	22.5	<0.2	<0.3	0.9	1.6
0358+202P07	03	58	12	+20	13.7	<0.2	<0.3	0.6	2.1
0359+169P07	03	59	52	+16	56.9	<0.2	<0.3	0.9	2.6
0412+024P07	04	12	11	+02	23.2	<0.2	<0.2	1.0	2.1
0413+023P07	04	13	40	+02	21.0	<0.2	<0.2	<0.6	2.1
0413+011P07	04	13	58	+01	03.8	<0.2	<0.2	<1.0	2.3
0417+008P07	04	17	40	+00	45.1	<0.2	<0.2	0.6	<1.5
0712+880P07	07	12	40	+87	57.8	<0.2	<0.4	0.9	<1.6
0845+515P07	08	54	16	+51	32.2	<0.2	<0.2	0.8	<1.4
0854+210P07	08	54	30	+21	00.4	<0.2	<0.3	0.9	<1.0
0855+108P07	08	55	59	+10	53.0	<0.2	<0.3	1.3	2.2
0902+128P07	09	02	33	+12	53.7	<0.4	<0.3	0.5	<0.7
0904+210P07	09	04	09	+21	00.2	<0.4	<0.3	0.5	<1.1
0910+234P07	09	10	58	+23	29.8	<0.2	<0.3	0.8	2.5
0915+511P07	09	15	08	+51	09.6	<0.2	<0.2	0.5	<1.3
0920+023P07	09	20	05	+02	19.6	<0.3	<0.3	0.6	<1.1
1010+865P07	10	10	21	+86	28.6	<0.2	<0.2	0.6	<2.0
1100+792P07	11	00	51	+79	15.6	<0.2	<0.2	0.8	1.5
1108+772P07	11	08	36	+77	12.9	<0.2	<0.2	0.9	2.1
1150+829P07	11	50	23	+82	52.8	<0.3	<0.2	0.5	<1.6
1157+860P07	11	57	35	+85	59.9	<0.2	<0.2	0.5	<1.6
1221+844P07	12	21	11	+84	26.7	<0.2	<0.2	0.6	<1.6
2359+846P07	23	59	08	+84	35.1	<0.2	<0.2	0.8	<1.6

The molecular biology of immunoglobulin D

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Immunoglobulin D (IgD) is co-expressed with immunoglobulin M (IgM) on the membranes of most B cells, yet its biological function has remained a mystery. Recent detailed information on the structure and transcription of the unusual IgD heavy chain (δ) gene in mouse suggests a complex genetic control. A model is presented for the developmental regulation of IgM and IgD and roles suggested for the membrane and secreted forms of IgD in the immune network.

VERTEBRATES respond to foreign substances by the production of antibodies, which are proteins consisting of two heavy and two light chains. In the higher vertebrates this response involves the sequential production of multiple classes of antibody with the same antigenic specificity but different effector functions. The effector or class of the antibody is determined by the constant (C) region of the heavy chain (C_H region). For the mouse, eight immunoglobulin classes have been identified: IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE and IgA; the H chains are designated with corresponding Greek characters and the genes encoding them are arranged in the order μ , δ , γ 3, γ 1 γ 2b, γ 2a, ϵ , α in a cluster spanning some 200 kilobase pairs (kbp) of DNA on chromosome 12 (reviewed in refs 1, 2). The light chains are encoded separately. The antigen-binding variable (V) region of the H chain is coded by a V_H gene that is assembled from separate V, D and J germ-line segments during B-cell ontogeny (for review see ref. 3). It is located to the left of the C_H cluster. A similar mechanism—but without D segments—applies to the light-chain variable region, which also contributes to antigen binding.

The primary response, elicited by the first exposure to an antigen, consists of antibody of the IgM class. The secondary response to the same antigen, however, involves the production of antibodies of different classes—IgG in serum and IgA in gut as well as IgM. Thus, between the primary and secondary responses, immune 'memory' for antigen is established. This is accompanied by a class switch in which the original V_H gene is expressed with a new C_H gene.

Antibodies are produced by B lymphocytes, each of which expresses immunoglobulin of unique antigenic specificity on its surface. Each immunoglobulin class can exist in both membrane-bound (m) and secreted (s) forms which differ in the carboxyl-terminal sequences of their respective H chains. One consequence of antigenic stimulation of a B cell is a shift of expression from the membrane-bound form to the secreted form which takes place without any change in either the specificity or the class of the antibody^{4,5}. Thus, the surface immunoglobulin serves as an external signal of what the B cell, if stimulated, will produce. This is crucial for three reasons: first, it ensures that secreted antibody elicited by the interaction of membrane immunoglobulin with antigen will be appropriately directed; second, it permits B cells of the secondary response (memory cells) to be regulated as separate pools; and third, it allows maintenance of a repertoire of potential responses in the resting B-cell population at little metabolic cost.

IgD behaves paradoxically in relation to the above simplified view of the humoral immune response. It is a predominant surface immunoglobulin on most B lymphocytes^{6,7}, yet it is not secreted on antigenic stimulation of the cells that bear it⁸. What

its role may be is a fascinating question, for elaborate mechanisms have evolved to make this immunoglobulin class behave oppositely to the others.

In this review we shall begin with a description of biological data about IgD and then summarize, from the molecular biologist's viewpoint, what has been learned about the δ gene. Next, the expression of the μ and δ transcription unit will be examined with an eye to understanding the mechanisms controlling RNA splicing in that system. Then, we shall make some suggestions as to how IgD may fit into the immune system. Although the predominant focus will be mouse, we shall also discuss the comparison between human and mouse structures, along with evolutionary implications.

Biology of IgD

IgD on the surface of B cells is co-expressed with IgM⁹⁻¹², but appears later^{13,14} and is considered to be an indicator of the mature or 'virgin' resting B cell. On a given B cell, membrane IgD has the same antigen-binding specificity as IgM^{15,16}. IgD antibody is a very minor component of serum (micrograms per ml compared with milligrams per ml for IgG)^{17,18}, so it seems unlikely that it has an important direct role in the neutralization of antigens. On the other hand, it can function as a receptor, for when heterologous antibodies against the μ or δ constant regions are used to mimic stimulating 'antigen', both stimulate replication¹⁹⁻²³. When the cell is stimulated either in this way or with mitogens^{24,25} or T-cell factors^{26,27}, IgM is synthesized and secreted whereas IgD production is shut off. This is consistent with the low levels of IgD but raises the question of how this regulation is achieved.

A controversial issue is the role of IgD in memory. Some hold^{28,29} that the majority of cells giving rise to the secondary response have lost IgD from their surface, whereas others argue^{30,31} that IgD loss is not a necessary stage in memory cell maturation.

Ablation of the population of B cells that carries IgD on their surface (IgD⁺) by administration of heterologous anti- δ antibodies to the animal continuously from birth does not abolish the immune response³². This contrasts with the profound immunodeficiency engendered by suppression of IgM⁺ cells with anti- μ antibodies^{33,34}. This difference may simply be due to the fact that IgM⁺ B cells are produced early in ontogeny and are precursors of most or all of the other cells involved in producing antibody, whereas IgD expression occurs after the B cell is competent to respond. IgD suppression *in vivo*³⁵ and anti- δ antibodies administered *in vitro*³⁶ do give rise to subtle shifts in the quantitative responsiveness of B cells to T-dependent antigens. This may indicate that IgD is involved in interactions

between T and B cells and in the regulation of B-cell populations.

Although the function of IgD remains obscure, some insight may emerge from the genetic and structural information that has recently begun to accumulate. These results reveal an unusual structure for the IgD protein, considerable complexity in its genetic regulation, and surprising species differences between man and mouse.

The unusual IgD protein

Through DNA sequence analysis we now know the structure of the δ gene and hence the amino acid sequence of the δ protein of the BALB/c mouse (summarized and compared with other H chains in Fig. 1). The outstanding feature of the molecule is that whereas the constant regions of other H chains have three or four domains, δ has only two³⁷. These domains, termed $C_{\delta 1}$ and $C_{\delta 3}$ (C_{H1} and C_{H3} in Fig. 1) by homology with other classes, are separated by a unique hinge segment which is quite long and lacks the cysteine residues that normally provide the links between the two H chains in the standard H_2L_2 subunit structure of immunoglobulin. However, both the membrane spacer region of the membrane-bound form of δ protein and the secreted terminus of the secretory form of δ protein have a cysteine which could cross-link the respective chains³⁸. The presence of both IgD monomers (H_2L_2) and IgD 'half-monomers' (one H chain and one L chain) on B-cell membranes, has been well documented^{39,40}, and there seems to be an equilibrium between the two forms on the cell surface. It is not known whether the secreted form exists in the same sort of equilibrium, although the H_2L_2 form has been demonstrated electrophoretically⁴¹. The transmembrane segment of δ is very similar to that of μ , and the carboxyl-terminal residues that extend into the cell are identical^{38,42}. On the other hand, the portions encoded by membrane exons that might lie just external to the cell-surface (spacers) differ radically in length and amino acid composition. This structural dichotomy may bear on whether antigenic stimulation through μ or δ gives the same cellular response.

The μ - δ gene locus

The detailed genetic map of the μ - δ region is shown in Fig. 2. The four constant region exons of the C_{μ} gene, including the secreted terminus (coded as part of the C_{μ} 4 exon and membrane terminal exons μ_m1 and μ_m2) are located within a span of 4 kbp of DNA⁴²⁻⁴⁵. The δ gene begins 2 kbp beyond and spans a much larger area (10 kbp)^{38,46-49}. The δ gene is unique in that both the membrane and secreted termini are separated from the body of the gene by a large (5 kbp) intervening sequence^{37,38,46,48}. In between δ_s and δ_m1 is a potential third carboxyl-terminal exon, termed δ_x (ref. 38), which has not been demonstrated to be translated into protein but which would encode a peptide with the hydrophilic characteristics of a secreted terminus.

In the course of B-cell differentiation these exons are differentially expressed. The first clue to how this is achieved came from analysis of B-cell tumours or hybridomas, which have served as models for resting B cells. The two lines studied in greatest detail are the BCL₁ lymphoma⁵⁰, which arose spontaneously in a BALB/c mouse, and the GLC28 hybridoma⁵¹, which was made by fusion of a hamster lymphoma to a mouse B cell. The BCL₁ tumour maintains many of the characteristics of resting B cells including the ability to secrete IgM after stimulation with lipopolysaccharide⁵². The GLC28 hybridoma retains characteristics of the hamster parent, including expression of hamster immunoglobulin⁴⁸, but both tumours clearly express murine IgM and murine IgD on the cell surface⁴⁸⁻⁵³.

In each of these cell lines, both μ and δ constant regions are expressed with the same V_H gene^{48,49}, and that V_H gene remains immediately 5' to the μ gene in cells producing IgD (see Fig. 2b). Thus, δ must be expressed from an RNA precursor containing both μ and δ and from which the μ gene is spliced out.

Myelomas

A well studied cellular model for the immunoglobulin secreting, terminal stage of B-cell differentiation is the myeloma. Analyses of many examples led to the discovery that the expression of the secreted forms of all immunoglobulin classes except for μ is invariably associated with the deletion of DNA (including μ), juxtaposing the expressed class with the V region. These deletions are mediated by recombination between 'switch sites'^{1,2} located immediately 5' of each of the C_H genes except for δ ^{54,55} (Fig. 2) and the process is termed class switch recombination.

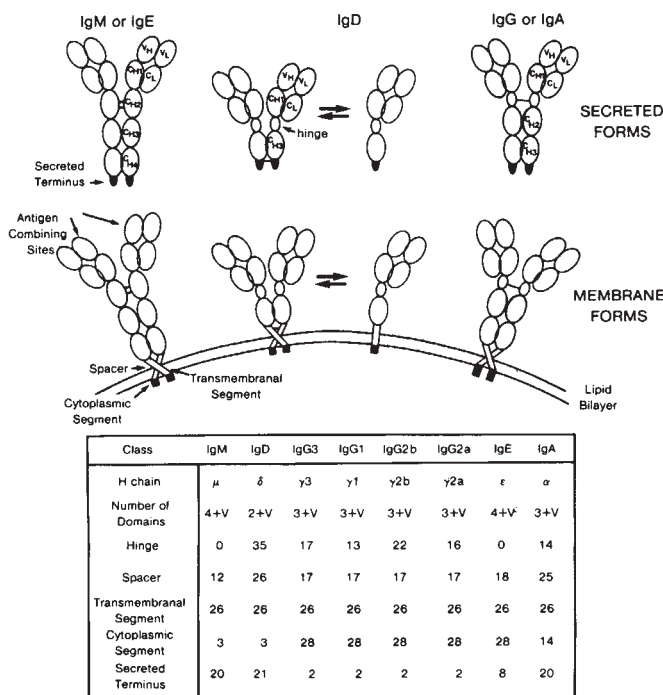


Fig. 1 Structure of membrane (m) and secreted (s) mouse immunoglobulins. The constant regions have from two to four domains depending on class, and in some cases a hinge segment is present between the first and second constant region domains. This is thought to introduce flexibility into the structure to allow the antigen binding site to achieve better contact with antigen⁹⁶. Shown at the top are the monomer s forms (H_2L_2) for the three structural types as well as the IgD half-monomer forms (shown in equilibrium with the monomer by arrows). Large and small ellipsoids represent domains and hinge regions, and dark ellipsoids represent the secreted carboxyl termini of the H chains. Non-covalent domain contacts are shown by the overlaps, and covalent H-H disulphide bonds by straight lines. Below, the three immunoglobulin types are illustrated attached to a B-cell membrane. Most of the molecule (including the antigen binding site constant regions and m-form specific spacer region) is on the outside of the lipid bilayer. The transmembrane segment spans the membrane and the cytoplasmic segment (box at the C-terminal end) protrudes inside. At the bottom of the figure is a structural summary for s and m forms of the eight mouse H chains. All numbers, except for domain numbers, refer to amino acids. Specific references used are: μ ^{42,43}, δ ^{37,38}, $\gamma 3$ (J. Wells *et al.*, in preparation), $\gamma 1$ ¹⁰²⁻¹⁰⁵, $\gamma 2b$ ^{103,104,106}, $\gamma 2a$ ^{105,107}, ϵ ^{108,109} and α ¹⁰⁹⁻¹¹⁰. [A discrepancy between the genomic^{37,38} and cDNA³⁷ sequences in the secreted terminus and an uncertainty in the position of the beginning of the $C_{\delta 1}$ domain have been resolved through analysis of peptide sequences⁹⁵ of murine δ protein. In other respects, the DNA sequence was confirmed by the peptide analysis. Thus the sequence of the myeloma protein can be considered to be quite solid. There is no proof that the secreted terminus of the myeloma protein is the same as the terminus of serum δ found in minute quantities in the normal animal. The structure of the membrane form³⁸ was established entirely from genomic DNA sequence using realistic, but unproven, assignments for the membrane exon boundaries. This structure has been confirmed in part by the demonstration that an anti-peptide antibody against the membrane spacer will precipitate δ -chain obtained from the surface of normal spleen cells (T. Shinnick, F.R.B. and P.W.T., unpublished observations). However, further protein analyses will be desirable to ensure that all splice boundaries have been correctly established.

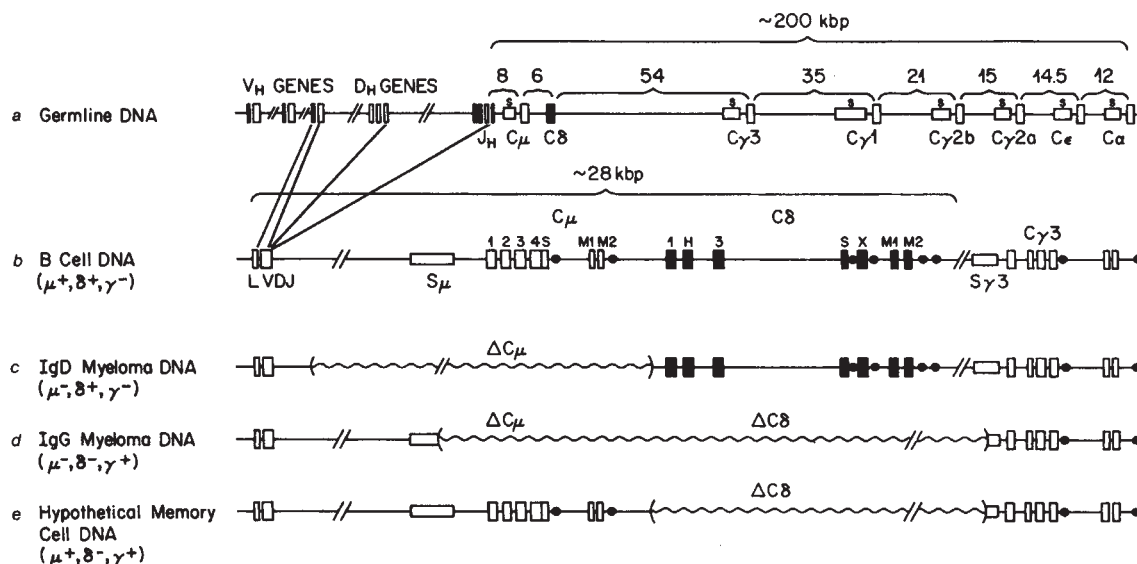


Fig. 2 Immunoglobulin gene arrangement and rearrangement during development. Tall boxes are protein encoding regions (δ regions are always in black), thin boxes are switch (S) recombination sequences, and dark dots are transcription (*end*) sites (see text). Unless indicated (//), distances are drawn to the scale. *a*, Map of the germ-line (unrearranged) H-chain locus. Distances, where indicated in kbp, are from centre to centre of a gene (for simplicity shown as a single box) or gene cluster. Switch sites are located 5' to all C_H genes except C_δ . *b*, Expanded map of the μ - δ transcriptional unit in B-cell DNA. As indicated by the lines from *a*, only the variable region segments (V_H , D_H and J) are rearranged in cells expressing IgM (μ -chains) or IgM and IgD (δ -chains) simultaneously. Here and in *c*-*e*, exon fine structure is shown (see text for details). *c*, IgD-secreting tumours delete (Δ) C_μ . The approximate length of the deletion (determined in this case from the myeloma TEPC 1017) is indicated by a wavy line, and the left and right recombination points are represented by parentheses. Note that this deletion does not utilize conventional switch sites (see text). *d*, IgG-secreting cells delete all genes between V - D - J and the expressed (in this example, γ_3) gene. Conventional switch sequences are utilized. *e*, Hypothetical DNA rearrangement for putative double-producing ($\mu^+ \gamma_3^+$) memory cells. The proposed deletion occurs at a recombination site 3' to C_μ and 5' to at least part of S_{γ_3} , thereby deleting C_δ and providing a reasonably short transcriptional unit for μ - γ membrane expression.

The switch site regions are composed of tandemly repeated pentamers: GAGCT mixed with GGGGT^{1,2,56} and similar variants. The boundaries of the deleted DNA are near, but not necessarily within, the switch site repeat^{1,2,57,58}. Despite the absence of a δ switch site, examples of IgD-secreting myelomas have been found^{41,46,59,60}. In these cases, deletion of C_μ has also occurred. In the one case so far examined, TEPC 1017 (ref. 55 and A. C. Gilliam, A. Shen, J. F. Mushinski, F.R.B. and P.W.T., in preparation), the deletion endpoints were not anywhere near sequences that resemble switch sites. Thus, in rare cases rearrangements can occur that activate IgD secretion but which are not mediated by switch sites. It is not clear, however, whether such deletion is related in any way to the biological mechanism of secretory expression of IgD. Since membrane IgD expression in B cells does not involve DNA deletion^{48,49}, we suspect the secretory expression is also controlled at the transcriptional level. Therefore, non-switch rearrangement may be an artefact limited to the transformed cells.

μ - δ RNA analysis

Additional information has been obtained by direct analysis of lymphocyte RNA. Table 1 lists the most important mRNAs and the cells in which they have been detected. The putative structures of precursors of some of them are shown in Fig. 3 (for details see figure legends).

Initiation of H-chain transcription begins at a promoter located just upstream of the active V gene⁶¹. So in a given B cell, as far as is known, all the H-chain RNAs have the same 5' end. The principal H-chain RNAs of the B cell are the membrane forms of μ and δ . In both the μ and δ myelomas, the secreted forms predominate, although membrane RNA is also synthesized. Several minor components not illustrated in Fig. 3 are described in the legends to Table 1 and Fig. 3. Thus, depending on its state of differentiation, the B cell can produce multiple forms of mRNA from the same DNA, each with a different 3' end and spliced in a unique pattern so that in some cases what is an intron in one RNA is an exon in another.

The donor and acceptor RNA splice sites at the ends of the immunoglobulin intervening sequences are typical of those of

other gene systems⁶² and immunoglobulin splicing appears to be no less accurate. Specifically, there is no noticeable tendency for the splicing apparatus to skip correct splice sites, thereby deleting exons from the message. This despite the fact that the μ - δ genetic region contains many sequences that are similar to the donor and acceptor splice sites of the exons which are not used as splice sites as far as is known. Control of splicing is thus as much a matter of avoiding incorrect splicing choices as of choosing the correct sites; and the pattern must be altered according to the state of cell differentiation.

Control of splicing

The study of adenovirus (reviewed in refs 63, 64) has shown that a critical step in determining differential expression of the late genes is the selection of the 3' end of the mRNA precursor. This is thought to occur by endonucleolytic cleavage of the precursor, with possible exonucleolytic trimming^{65,66}, followed by addition of polyadenylate [poly(A)] residues to the 3' end. RNA splicing to remove intervening sequences generally follows both clipping and polyadenylation^{63,65,67}.

It is not known what structural feature of the precursor RNA determines the position of 3'-end clipping, so we have adopted the neutral term '*end site*' for this hypothetical complex recognition element. The sequence AATAAA⁶⁸ or AATTAAA⁶⁹ that occurs near the 3' end of most eukaryotic mRNAs may be a component of the recognition site. However, these sequences occur at 16 places in the μ - δ region and only 6 are (apparently) used (J. E. Richards, unpublished data). The locations of the six functional *end sites* of the μ and δ transcripts are indicated in Figs 2-4.

It has been proposed that control of expression of the membrane as opposed to the secreted forms of IgM⁴², as in adenoviruses, is controlled at the level of 3' end selection (*end*₁ versus *end*₂ in Fig. 3). In support of this model, a recent analysis⁷⁰ of nuclear RNA in early B-cell tumours has directly demonstrated the first two predicted μ precursors of Fig. 3.

Choice of splice sites may follow automatically from choice of *end sites*. In the case of μ , the splicing choices are simple. The last three splice sites are missing from the shorter transcript

(Fig. 3), so they cannot be used and the S terminus is coded as an extension of the C_{μ} 4 exon. The choice in the IgD myeloma TEPC 1017 is also simple. end_1 and end_2 are deleted from the DNA (Fig. 2c), thereby allowing straightforward expression of the δ mRNA from end_3 . Membrane or secreted IgD expression in B cells is more difficult to understand for it requires removal of μ sequences from a long δ mRNA precursor containing both μ and δ exons. Thus the splice sites of μ , which must not be skipped in the μ transcript, most definitely must be skipped in order to produce δ mRNA.

In these more complex cases, how might the selection of splice sites be made? One possibility would be a developmentally regulated change in the specificity of the splicing apparatus, but this assumes sequence differences in the splice sites that might be differentially recognized, and a computer search (F.R.B., unpublished) has not suggested such differences. We would therefore like to propose that RNA precursors of different lengths might adopt different secondary structures, so exposing alternative splice sites to the splicing machinery. The developmental regulation of heavy-chain gene expression could in this case be simply a matter of regulation of 3' end site selection. According to the model of Fig. 4, 3' end site selection could be regulated simply by alterations in the level of the enzyme ('endase') that cuts the RNA precursor at the end site. Since an RNA molecule must survive a cross fire of endonuclease cutting to reach full length, we dub this the 'gauntlet' model. We assume that endase is present in the resting B cell in limiting amounts. The level of expression of various 3' ends in B cells, then, would depend on the relative affinities of different end sites for endase. To account for the mRNA abundances discussed above and in the legend to Table 1, we have postulated the relative strengths of end sites—as shown in Figs 3 and 4.

In the differentiation of a B cell to a secreting cell, we propose that the quantity (or activity) of endase is increased. The effect would be to force the usage of more 5' (promoter-proximal) end sites, regardless of affinity, because the first cut defines the end of the RNA regardless of any downstream cuts (gauntlet effect). In the limit this would drive the system to produce only the shortest possible precursor RNA in the fully differentiated plasma cell. It would also permit a continuous progression from resting B cell to plasma cell rather than an abrupt jump, which could help explain how IgM antibodies can be secreted by B cells (blasts) that have not become fully differentiated into plasma cells. The model also explains why, in all myelomas, the most strongly expressed mRNA is the one that is defined by the first end site in the DNA after the promoter, and why the switch site recombination is needed to obtain efficient expression of downstream classes in myelomas.

Other observations which this model helps to explain are: (1) The wide and continuous variations of μ to δ ratios observed in B-cell populations^{71,72}. (2) The transitory nature of IgD expression on the resting B cell and its disappearance on activation²⁴⁻²⁷. (3) The observation (ref. 73 and D. J. Kemp, personal communication) of an RNA fragment containing only μ_m sequences whose size corresponds roughly to the end_2 to end_3 interval. Such ' end - end ' fragments would be expected if the RNA polymerase continues down its DNA template beyond the first end site, as suggested in adenovirus^{63,64}, and the endase nicks the downstream end sites as well. (4) The observation⁷⁴ that neonatal B cells seem to produce some secreted IgM. This could be due to an intermediate level of endase in these very early B cells.

An aspect of immunoglobulin expression that is not easily explained by the gauntlet model is the expression of downstream classes (IgG, IgE or IgA) in memory B cells, if these are coded from an RNA precursor up to 200 kbp in length as has been reported⁷⁵.

Selection of 3' ends by cutting of RNA precursors at end sites clearly is only one of several levels at which immunoglobulin expression is regulated. Others include control of transcription initiation, possibly involving enhancer-like elements⁷⁶⁻⁷⁸, transcription termination and chromatin structure alterations⁷⁴.

Table 1 Classification of μ and δ mRNAs

3' Terminus	Size (kb)	Name	Occurrence
end_1	2.4	μ_s	Spleen and B-cell tumours (weak), μ myelomas (strong)
end_2	2.7	μ_m	Spleen and B-cell tumours (strong), μ myelomas (weak)
end_3	1.75	δ_s	δ myelomas (strong)
end_3	3.2	δ_s	Spleen, B-cell tumours and δ myelomas (very weak)
end_4	2.65	δ_x	Spleen and B-cell tumours, (weak) δ myelomas, (very weak)
end_5	2.1	$\delta_{m-minor}$	Spleen and B-cell tumours (weak), δ myeloma (very weak)
end_6	2.9	$\delta_{m-major}$	Spleen and B-cell tumours (strong), δ myelomas (weak)
end_6	2.9-	$\delta_{m-major} + 3'$ heterogeneity	Spleen and B-cell tumours (very weak), δ myelomas (very weak)
end_2	2.0	μ_m 3' fragment	μ myelomas and T cells (very weak)

Sizes of the end_3 , end_5 and end_6 δ mRNAs listed above^{59,98} were also measured as 1.6 (ref. 47) and 1.8 (ref. 48), and 2.7 (refs 47, 48), respectively. The 2.4-kb μ_s and 2.7-kb μ_m mRNAs that were originally described in IgM-secreting myelomas^{42,44,45} correspond to end_1 and end_2 . These RNAs are also observed in splenic B cells⁹⁹. The quantity of μ_s form mRNA is considerably reduced when large spleen cells (blasts and plasma cells) are selectively removed (D. Yuan, unpublished observations). A number of 'sterile' μ RNAs, lacking some exonic component required for normal translation, have been observed in the cytoplasm of both B and T cells^{100,101}. In all δ^+ or $\mu^+\delta^+$ cells examined^{48,59,98}, two forms of δ mRNA are found, which we term here as $\delta_{m-minor}$ and $\delta_{m-major}$ according to their relative abundance. These terminate at end_5 (~2.1 kb) and end_6 (~2.9 kb), respectively. The 10–50-fold difference in level of expression between these forms^{48,98} may be due to differences in the strength of termination. These mRNAs apparently code for the same protein³⁸ and differ only in the length of the 3'-untranslated region. The $\delta_{m-major}$ form also shows 3' heterogeneity caused by removal of intervening sequences within the 3' untranslated region of some molecules but not others^{38,48}.

The 1.75-kb transcript for δ_s , ending at end_3 , is strongly expressed only in the IgD-secreting tumours from which the μ gene has been deleted at the DNA level^{47,48,59,98}. In spleen cells and B-cell tumours, the only RNA that can be found ending at end_3 is a 3.2-kbp species⁹⁸. This species, however, is a true B-cell product since it is enriched in the small B-cell population (D. Yuan and P.W.T., unpublished results). No information is yet available as to its splicing pattern, nor is it clear whether this RNA codes for δ_s of the myeloma type. Also found in spleen and B-cell tumours is an RNA of 2.65 kb ending at end_4 (refs 38, 98). This RNA may code for δ_x , although there is no evidence for this terminus being used in a protein. Either the 3.2-kbp or 2.65-kbp RNAs might be unspliced precursors and thus might not be *bona fide* mature mRNAs.

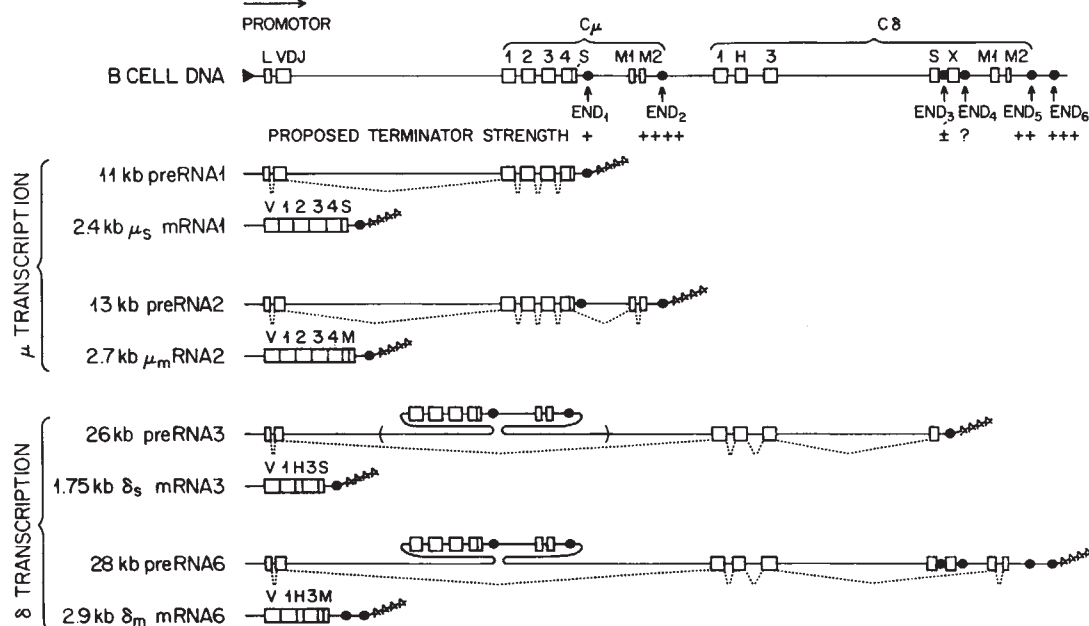
There is also an indication that post-translational regulation occurs in this system, since 10-fold less δ mRNA than μ is found in the cytoplasm of splenic B cells which have equal amounts of the Htwo proteins on their surfaces⁸⁰.

Elucidation of the relative importance of these mechanisms will require detailed analysis of RNA in a variety of types of cells. We have recently observed substantial δ specific RNA in nuclei of neonatal B cells that are expressing μ but not δ on the surface (unpublished data of D. Yuan and P.W.T.). This RNA could reflect end - end fragments as predicted by the gauntlet model. Such RNA is not found in myelomas committed to IgM, however. This may indicate that transcription termination at a site between μ and δ comes into play in fully differentiated plasma cells.

The role of IgD

IgD is different from other immunoglobulins in structure, exon arrangement, physiological localization and in its behaviour in the immune response. Yet we have no idea of its function.

Fig. 3 Transcription and splicing patterns of major μ and δ mRNA precursors. A detailed scale map of the μ - δ transcriptional unit including the promoter ($\blacktriangleright$) is given at the top (also see Fig. 4 legend). The relative strengths, based on mRNA abundances (see Table 1 legend) of transcription *end* sites are indicated below each one with +. RNAs are shown as precursor (preRNA)-product mRNA pairs. Sizes of the preRNAs are predicted from the DNA sequence, assuming that 3'-end termination results from RNA cleavage (see text for details). Sizes of the mRNAs have been determined experimentally. Splicing is indicated by dotted lines and poly(A) tails are shown as AAAAA. Splicing of the μ - and δ -containing preRNAs 3 and 6 to yield exclusively δ mRNA products may depend on eliminating the μ sequences from the splicing complex through local secondary RNA structures (simplistically illustrated in the figure by a total looping out of μ). In δ -expressing myelomas, the C_μ gene and its associated *end* sites are already removed by DNA deletion (denoted by parentheses on the preRNA3). See text for references.



Perhaps it simply serves as an auxiliary antigen receptor on the B cell^{22,23,81}, but this seems a rather trivial explanation for the evolution of such a complex system. We prefer to suppose that it has a role in regulation of B-cell populations, because this would help explain the paradoxical aspects of IgD expression. (A third possibility, of course, is that it has no function.) Since IgD appears on the mature virgin B cell and decreases when the B cell is stimulated by antigen, the virgin cell would be the one in whose regulation IgD would be likely to participate.

These cells arise by differentiation from stem cells of the bone marrow or fetal liver in a process that involves random generation of variable regions from the germ-line *V* gene segments, thus producing cells with the antigenic specificities that represent a sampling of the entire repertoire of *V_H* regions that can be constructed from the germ line. B cells have an average lifetime of a few weeks, so their population is constantly turning over. The pool is depleted by cell death and fed by the influx of virgin cells plus replication of cells that may be stimulated by antigens or other regulatory influences which may be responsible for maintenance of immunological memory at the B-cell level. One way of accounting for the persistence of memory cells is through the network theory⁸², in which antibodies themselves function to regulate the distribution of *V* regions represented in the B-cell population by binding to idiotypic determinants of surface immunoglobulin on B cells so as to affect differentially the replication of B cells expressing various determinants. Class-specific and/or idotype specific factors produced by T cells play the part of regulators in modern formulations of the network theory⁸³⁻⁸⁶.

IgD and especially its spacer segment (Fig. 1 and ref. 38) would be well suited to serve as a receptor on the B cell for the class-specific component of the network influences regulating the primary response. Since IgD expression is shut down on antigenic stimulation, this marker could serve to focus these influences specifically onto the virgin B-cell population. Presumably, the spacers of other membrane immunoglobulins could serve the same role for regulation of the other classes. The reason the spacer in particular is proposed for this function is that it is encoded on the same exon as the transmembrane segment (also true of other immunoglobulins). Thus, this determinant cannot appear on the surface of a cell by cytoplilic adsorption of serum antibody nor could serum antibody compete for sites with a factor directed against the spacer portion.

It is more difficult to account for the secreted form of IgD (either δ_s , δ_x or unknown terminus). However, secreted IgD could complete a feedback loop by supplying afferent signals representative of all the idiotypes present in the B-cell repertoire to the T-cell population. This role would fit with the prediction of the gauntlet model that the s form of IgD would be secreted from resting B cells, not IgM-secreting ones. The low stability of serum IgD²² would also serve a function, since an intercellular messenger molecule should not have an excessive half life if it is to function properly in that role. There is, in fact, some evidence that a factor emitted by B cells is needed for the development of a class of isotype-specific *T_H* cells⁸⁷. This factor might be the s form of IgD.

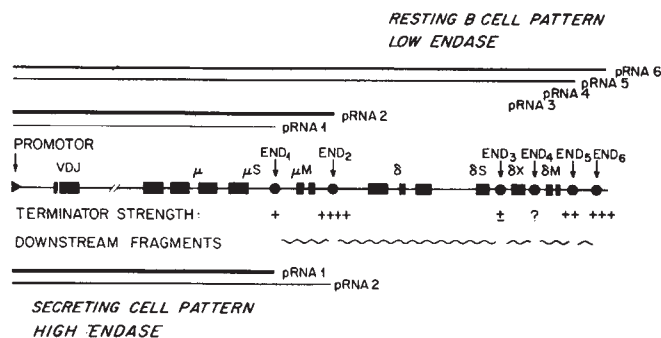


Fig. 4 The gauntlet model for modulation of μ - δ transcription. The μ - δ B-cell transcriptional unit is shown in the centre with exons as boxes and 3' *end* sites as dots (see Fig. 3 legend). All primary transcripts (preRNA 1-6) initiate at the promoter ($\blacktriangleright$) left of *V-D-J* and terminate at *ends* 1-6 as indicated by the relative length of the lines. The relative abundances of the preRNAs, reflected by experimentally observed abundances of their mRNA products (Table 1 and its legend) are indicated by the thickness of the lines. The model predicts that in the resting cell (top of figure), the enzyme (endase) that cleaves the preRNAs at *end* sites is in low concentration. Since fewer molecules are cleaved, longer transcripts (membrane forms) would predominate with their relative abundances weighted by *end* sequence affinities (+) for endase. On activation to secretion (patterns at the bottom of the figure), the endase activity and/or concentration is increased dramatically, resulting in almost complete dominance of promoter-proximal *end* sites regardless of affinity. The wavy lines represent hypothetical *end-end* fragments.

Human and mouse IgD

The human δ protein chain, sequenced from myeloma material⁸⁸⁻⁹¹, does not show all of the oddities of the mouse molecule. The human chain is conventional in having three rather than two constant region domains. Its hinge is even longer than the murine one and contains a bizarre cluster of charged amino acids in its C-terminal portion, as well as a cysteine residue that could be used for normal inter-H-chain linkage.

The sequence match between murine and human IgD molecules is poor in the first domain (~33% amino acid match). Only the third domain, exclusive of the secreted terminus, has the high degree of homology (52%) expected for corresponding structures.

The human δ gene has been cloned and a fragment sequenced to show that its position in human DNA is analogous to that in the mouse⁹². However, the disappearance of an entire domain in the mouse clearly represents a dramatic evolutionary difference that raises a serious question about whether the two molecules could serve the same function (or any function) in the two species. Could the apparent loss of a domain result from a technical artefact? We think that this is unlikely because data for rat^{93,94} and another strain of mouse⁹⁵ indicate that both

have the same two domain δ -chain structure that we deduced for BALB/c^{37,38}. Moreover two independent human δ -chains have been sequenced with identical results. It seems that there must have been a dramatic divergence in the δ gene after the separation of human from rodent evolutionary lines.

It is unclear how these differences should be interpreted. Logic would indicate that the second domain is not important to IgD function, and would focus attention on the third domain or possibly the spacer segment, whose sequence is unknown in man. On the basis of recent studies of domain deletion mutants (V. Oi, personal communication), there is reason to believe that the absence of a C₂ domain may not cause extensive disruption of general immunoglobulin folding patterns⁹⁶. In any event, the existence of IgD in seven species (ref. 97 and refs therein) probably indicates that the molecule must have conferred a significant advantage over evolutionary time.

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Palaeoanthropological discoveries in the Middle Awash Valley, Ethiopia

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A survey of Ethiopia's Middle Awash Valley in 1981 yielded new and significant archaeological, palaeontological, geological and geochronological results. Included in the new discoveries are hominid fossils from the Pliocene which show primitive cranial anatomy and a femur adapted to bipedality. Archaeological records document human technological change spanning the past 1.5 Myr.

RESEARCH into Africa's rift system has elucidated critical stages of human technological and physical development, providing data on environments, behaviour and phylogenetic relationships of extinct hominid species. The 1981 survey of Ethiopia's Middle Awash Valley resulted in new discoveries and confirmed the importance of the region for human origins research.

The study area is situated along the Awash River Valley between the site of Hadar in the north and the town of Gewane in the south. Miocene, Pliocene and Pleistocene deposits crop out along both eastern and western valley margins as well as in the basin centre. Geologist Taieb discovered the palaeoanthropological significance of the region during geological reconnaissance in the 1960s¹. In 1971 and 1972, Taieb, Johanson, Coppens and Kalb conducted a preliminary survey of the central Afar. They formed the International Afar Research Expedition (IARE) and began to investigate Hadar. Discoveries there yielded new insights into hominid evolution^{2,3}.

The Middle Awash was explored between 1975 and 1978 by Kalb's Rift Valley Research Mission in Ethiopia (RVRME). Small excavations were done and a partial hominid cranium was found in Middle Pleistocene deposits at Bodo⁴. Taieb's initial work had identified sediments along the Awash ranging between Pliocene and Quaternary^{1,5}. The RVRME conducted the initial mapping and palaeontological work here. Biostratigraphical data indicated prolonged deposition⁶⁻¹⁰.

Wendorf, an RVRME member, invited one of us (J.D.C.) to join the archaeology team in 1977 and in 1981 the Ethiopian Ministry of Culture invited J.D.C. to lead a multidisciplinary team to the Middle Awash. Two months were spent in the field examining localities in the western, central and eastern sectors of the study area. Abundant archaeological remains ranging from later stone age to Oldowan were identified. Biostratigraphical analysis confirmed that approximately 6 Myr of vertebrate evolution is represented within the study area. A programme of tephrostratigraphy was initiated and the first radiometric dates for the Middle Awash strata were obtained.

Stratigraphy

Exposure of sediments in the area is often good. However, lateral correlation is hindered by common NNW-SSE and NNE-SSW normal faults that dissect the deposits and bring rocks of different age into fault contact. Lithological correlation is further impeded by erosional gaps within the sequences and between the areas. Lateral facies changes and the repetitive nature of

the late Neogene sedimentation complicate the geology. For these reasons the numerous volcanic horizons provide essential stratigraphical and chronological control. Kalb *et al.*^{7,9} recently formalized sedimentological groups, formations and members for the entire Middle Awash area. We refer to these units below but prefer to work with the previously introduced, informal stratigraphical nomenclature⁶ until tephrostratigraphy clarifies formational boundaries in the region. Our 1981 survey showed that an east-west geological transect across the Bodo and Dawaitoli areas provides a useful guide to the general stratigraphical and tectonic relationships of Plio-Pleistocene deposits of the eastern sector of the Middle Awash (Figs 1 and 2). The 1981 archaeological and palaeontological discoveries reported here are either within or firmly correlated to the stratigraphical sequence at Bodo.

Deposits that crop out closest to the Awash in the eastern sector consist mostly of sterile brown clays and silts but include additional interbedded strata in the Bodo, Dawaitoli, Hargufia and Meadura catchments. These strata, the Upper Bodo Beds of Kalb *et al.*⁶ are particularly rich in Middle Pleistocene artefact assemblages and fauna. Work on volcanic horizons in these beds should help to clarify relationships between the Bodo, Meadura and Dakanihyalo members of the Wehaietu Formation formalized by Kalb *et al.*⁹.

Lower Pleistocene sediments found in the Bodo area are structurally separated from the Middle Pleistocene outcrops by normal faults, downthrown to the west. Similar structural relationships are observed between Pliocene and late Pliocene/Lower Pleistocene rocks at Dawaitoli, Wilti Dora and Matabaietu. The Lower Pleistocene beds at Bodo, the Middle Bodo Beds of Kalb *et al.*⁶ (Matabaietu Formation⁹) consist of brown clays interbedded with both unconsolidated and calcite-cemented sands and gravels. The cemented sands are richly fossiliferous and at least three volcanic tuffs are interbedded in the sequence.

Pliocene deposits exposed in the Bodo area are >70 m thick and constitute the Lower Bodo Beds of Kalb *et al.*⁶ (Sagantole Formation, Beearyada and Kalaloo Beds⁹). Seven tuffaceous units were identified in the composite section and one of them, informally termed the Cindery Tuff (CT), was used as a primary chronostratigraphical marker horizon (Fig. 2). This tuff is a 15-30-cm thick, grey lapilli tuff. It is a primary plinian ash deposit that, in hand sample, shows grey pumice clasts, clear grey glass shards and scoracious black glass fragments ranging from sand-size particles (1.0 mm) to fine, granule-size particles

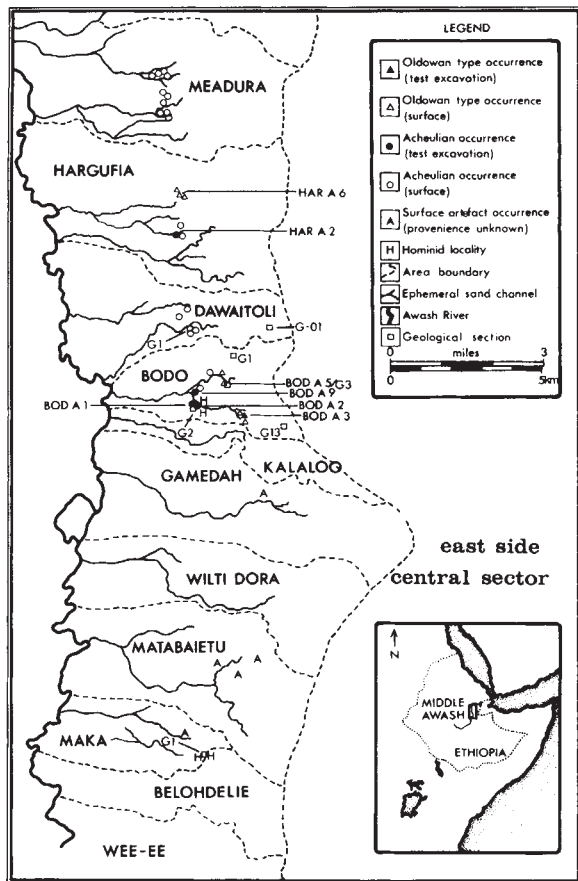


Fig. 1 The central eastern sector of the Middle Awash study area.

(up to 5.0 mm). The CT crops out extensively at Bodo and appears to have erupted into a very shallow, laterally extensive, freshwater lake, probably not highly alkaline. The description of "... a widely traceable coarse or lapilli tuff (10–35 cm)" at the base of the Kalaloo Beds^{7,9} is here interpreted as the only previous description of the Cindery Tuff. Mammalian footprints found impressed into the upper CT surface at Gamedah suggest shallow lake conditions, possibly the lake margin. We have already traced the CT over a distance of ~30 km from Wee-ee to Meadura (Fig. 1).

Petrographic studies and microprobe geochemistry show that the CT is a primary ash deposit, not reworked, and containing no obvious detrital phases. The ⁴⁰Ar/³⁹Ar analyses performed on CT feldspars and glasses are concordant, yielding ages of 3.9–4.0 Myr. These results are corroborated by preliminary zircon fission track age estimates conservatively set at 4.0 ± 0.2 Myr. An isotopic age concordance of 4.0 Myr for the CT is thus a reasonable assessment of its eruptive age, and age consistent with biostratigraphic findings of the 1981 fieldwork and previous analyses^{12,13}.

Outcrops exposing the CT are among the most laterally continuous of this age known in Africa. At least 50 m of horizontally bedded silts, clays, diatomites, lenses of fossiliferous sand and gravel and occasional bands of volcanic ash, limestone and lignite lie below the CT. The sections beneath the CT are lithologically uniform from Wee-ee to Bodo. Diatomite is common in the upper 20 m of sediments beneath the tuff where 1–6 m thick diatomite beds alternate with grey-brown clays of comparable thickness. Facies changes are more common above the CT. Approximately 20 m of sediment overlie the CT in the Maka area. These include a vitric tuff and fossiliferous, yellow-orange stained gravel-sands. The cross-bedded sands and gravels alternate with subhorizontal silts and clays above the CT and indicate a major contrast to deposition below the tuff—a contrast which persisted during Lower and Middle Pleistocene times.

Tectonic events were probably responsible for the demise of Miocene and early Pliocene lakes in the area¹¹.

Archaeology

Taieb¹ was the first to record artefacts coming from Plio-Pleistocene sediments in the central Afar. Kalb *et al.*^{8,14–16} have also reported the existence of numbers of archaeological sites in the Middle Awash yielding stone artefacts of Oldowan, Acheulian, Sangoan and later age and have published accounts of two of these^{15,17}. Corvinus and Roche^{18,19} recorded Oldowan-type artefacts from conglomerates of the upper beds of the Hadar Formation in the Gona River Area. Harris²⁰ has subsequently test excavated and described an occurrence of Oldowan artefacts and bone *in situ* in fine-grained sediments in the Gona catchment; these, it is suggested, might be nearly 2.4 Myr old.

Our own survey concentrated on sediments of Upper Pliocene (Lower Bodo Beds⁶; Matabaietu Formation⁷) and Lower and Middle Pleistocene (Middle and Upper Bodo Beds⁶; Wehaietu Formation⁷) ages, situated on the east side of the Awash between the Maka and Meadura catchment areas and also at Dakanihyalo on the west side of the study area. Oldowan assemblages were found eroding from overbank silts in the Middle Bodo Beds⁶ exposed in the Ounda Bodo catchment. Typologically Lower Acheulian occurred in a lag gravel capping Pliocene sediments at Maka and in fossiliferous fluvio-lacustrine sediments at Dakanihyalo. Numerous occurrences as previously reported by Kalb *et al.*⁸ were found eroding from Middle Pleistocene (Upper Bodo beds⁶; Wehaietu Formation⁷) channel sands and silts. Two of the Oldowan and four of the Acheulian sites were test excavated.

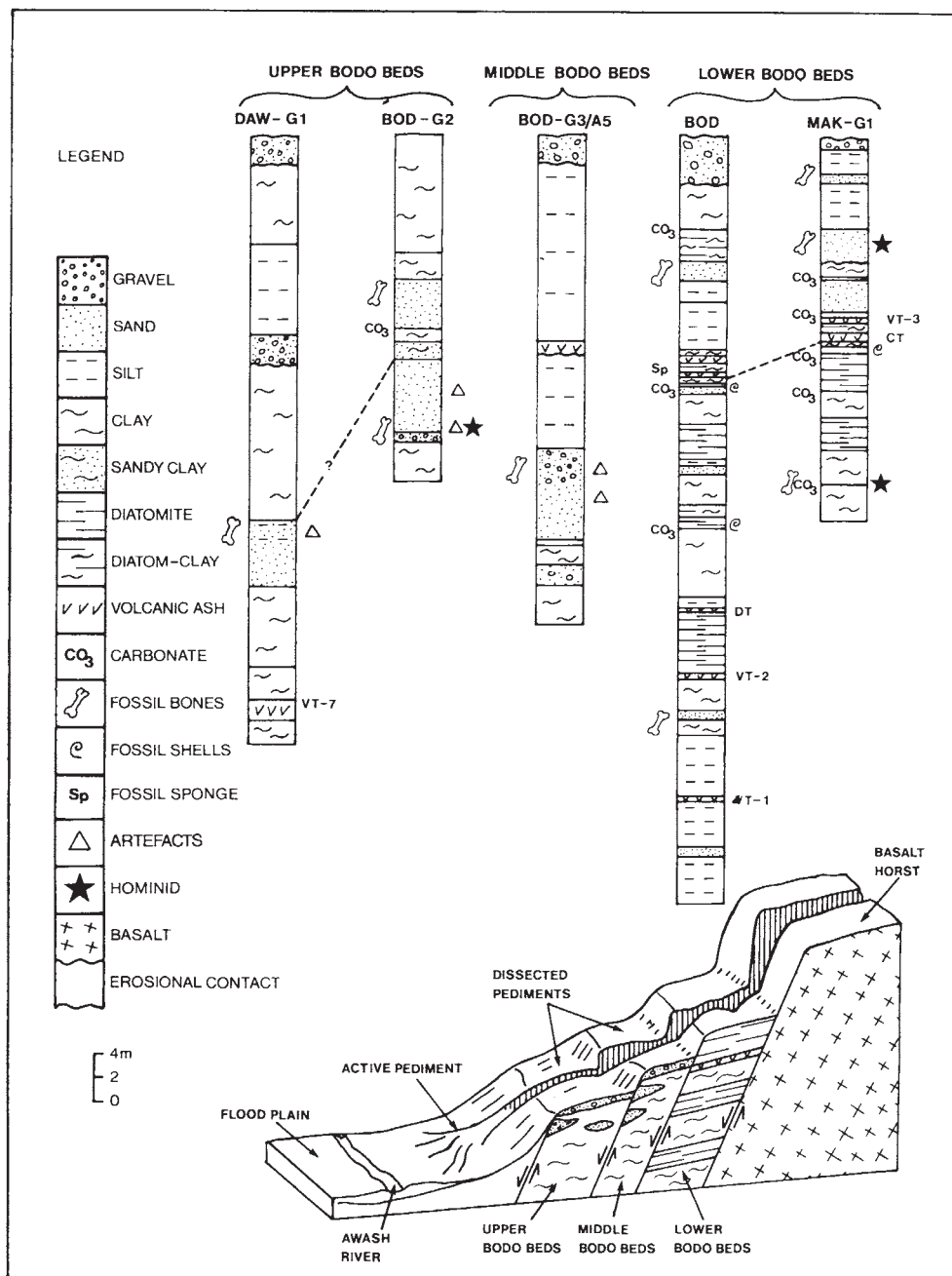
Oldowan

Kalb *et al.*⁸ have reported Oldowan artefacts eroding from Lower Pleistocene sediments at Wilti Dora but, in spite of close search, we were unable to locate any *in situ* artefacts although heavily patinated choppers and flakes traced to an overlying lag gravel lie on the slopes in some places. At Matabaietu, where a surface Oldowan occurrence had previously been reported¹⁵, the 1981 survey found small numbers of heavily patinated artefacts on the surface and close to the bones of elephant and hippopotamus. This association could be fortuitous resulting from deflation so that, until stratified archaeological occurrences are discovered and excavated, the designation of Matabaietu as an Oldowan locality must remain uncertain.

We were, however, fortunate in locating in the Bodo drainage five Oldowan occurrences. These are the earliest stratified occurrences of stone artefacts found in the Middle Awash so far, although older occurrences can be expected. The assemblages, characterized by small cores and flakes with occasional retouched pieces, show morphological and technological similarities to Plio-Pleistocene stone artefact occurrences assigned to the Oldowan Industrial Complex found just to the north at Hadar and elsewhere in the rift system (Fig. 5, nos 7–13)^{18–22}.

The Oldowan sites are clustered in two fault-bounded blocks of sediment constituting the Middle Bodo Beds⁶. The northern block contains fauna which indicate an age of 1.3–1.5 Myr. The stratigraphical relationships of the archaeological occurrences in the northern and southern blocks at Bodo remain to be worked out in detail. The most informative site, BOD-A3, is situated in the south-east sector, in the Ounda Bodo catchment. A total of 389 stone artefacts and numerous fragmentary faunal remains were found in relatively fresh condition scattered over a steep-sided outcrop of fine-grained fluvial deposits. Surface artefacts include several flake scraper forms ($n = 10$) as well as modified and utilized flakes and cobbles including hammerstones ($n = 8$). The major proportion of the assemblage ($n = 371$) is cores, unmodified flakes and flaking debitage. Another 45 stone specimens were recovered *in situ* from a 10 m² excavation. Fine-grained basalt was the preferred raw material but artefacts were also made on glassy or aphyric rhyolite, welded ignimbrite and chert. Most faunal specimens are highly fragmentary and

Fig. 2 Representative geological columns showing the three main stratigraphical elements present at Bodo. Also shown are the stratigraphical positions of artefacts and the three fossil hominids found in 1981. The Pliocene section at Bodo is a composite of three separate measured sections (DAW-G01, BOD-G1 and BOD-G13, see Fig. 1) that were correlated in the field using the Cindery Tuff (CT) or Doublet Tuff (DT) as stratigraphical markers. An idealized cross-section is presented which illustrates the general geomorphic, stratigraphical and structural relationships between the main stratigraphical elements in this area.



the long bone fragments display longitudinal and spiral fracture. No cutmarks were observed. The fresh condition, lack of size sorting, lack of preferred orientation and sedimentary matrix of the specimens indicate a low-energy environment of silty overbank deposition suggesting an occurrence in minimally disturbed context.

Several similar archaeological occurrences were found nearby in overbank silty deposits. Close to one of these, designated BOD-A4, clay samples were collected from a cone-shaped, reddish area. Magneto-thermal analyses by M. Barbetti at the University of Sydney Radiocarbon Laboratory showed that the BOD-A4 clay samples had been baked. Such baked clay concentrations occur in sediments ranging in age from Pliocene to Middle Pleistocene in the Middle Awash. Stone artefacts and fractured bone occurred adjacent to some of these concentrations in the Pleistocene sediments while others again had nothing associated. Two Middle Pleistocene occurrences were sectioned and found to consist of a cone-shaped mass of burnt clay with a shallow concave base. Channels in some of these burnt concentrations are evidence of insect activity, probably termites. These features have the appearance of the burnt earth that is built up round the bases of old trees and tree stumps that have

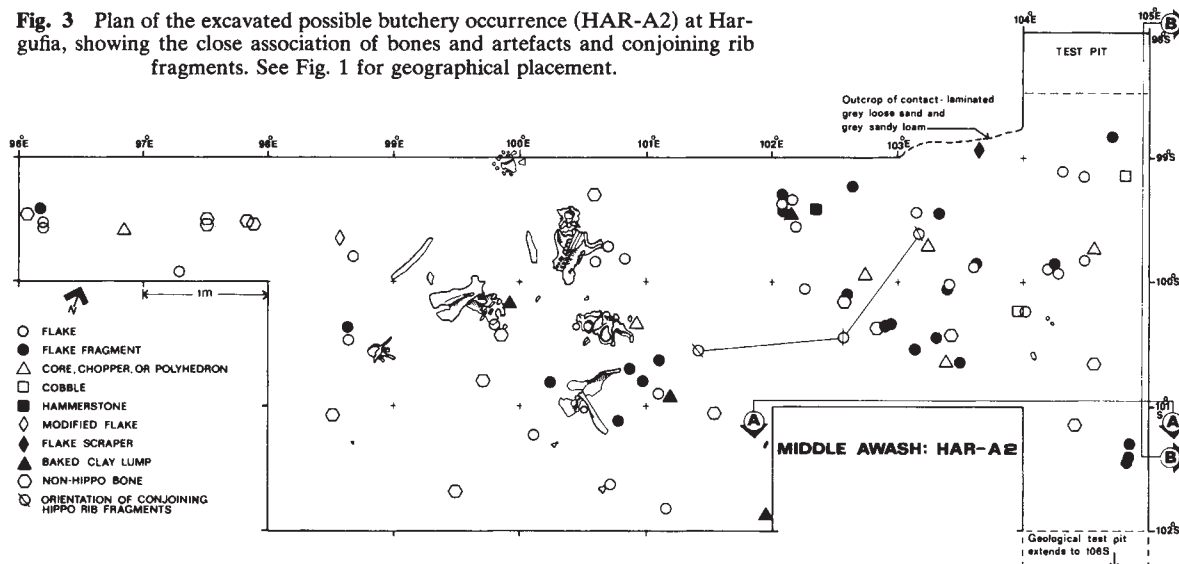
burned and are commonly to be seen in the tropics today. While it seems likely that many of these occurrences resulted from 'natural' fires, the observation made by one of us (J.D.C.) in central India early in 1982 of the way in which tree stumps were set alight intentionally by farmers, in order to have fire always available when they were working in areas distant from the homesteads, makes it possible, so great is the attraction and significance of fire for the human race, that early hominids might similarly have used and preserved fire in such a manner.

In view of the controversy surrounding the controlled use of fire by early hominids^{23,24} and due to the fact that these localized, discoloured areas are a widespread phenomenon in the Middle Awash, a systematic study of these features to determine more precisely the nature of each occurrence, temperature ranges, palaeomagnetic field strengths, the agencies that caused the baking and the extent to which artefacts and bone may be significantly associated should be given high priority in the future.

Acheulian and developed Oldowan

The two occurrences that can be assigned to an earlier stage of the Acheulian Industrial Complex—from Maka and

Fig. 3 Plan of the excavated possible butchery occurrence (HAR-A2) at Hargufia, showing the close association of bones and artefacts and conjoining rib fragments. See Fig. 1 for geographical placement.



Dakanihyalo—are made from lavas and comprise thick-butted and sometimes triangular-sectioned bifaces, core-choppers and flakes technologically and typologically analogous to Lower Acheulian assemblages from East Africa^{22,25}. The fauna associated with the Dakanihyalo assemblage as well as the technological and typological attributes of the artefacts suggest, therefore, that the Dakanihyalo Member of the Wehaietu Formation may antedate, rather than postdate, the Bodo and Meadura Members^{7,8}.

On the east side of the Awash, deposits (Upper Bodo Beds⁶; Wehaietu Formation⁷) that outcrop closest to the river consist mostly of sterile brown clays and silts but interbedded strata in the Bodo, Dawaitoli, Hargufia and Meadura catchments are particularly rich in artefact assemblages that we ascribe to the Upper Acheulian and developed Oldowan industrial complexes. Those occurrences least disturbed by fluvio-lacustrine resorting or other agencies are found within massive silts and clayey fine sands which often merge laterally into coarse channel sands and

gravels. Where stone artefacts are found within channel sands they may be imbricated but have not necessarily been moved very far.

Four test excavations were carried out in 1981. Assemblages are mostly of two kinds—either (1) large concentrations of Acheulian handaxes and cleavers with flaking waste but a significant paucity of light-duty artefacts and fauna (for example, BOD-A2) or (2) developed Oldowan-type assemblages of flakes (some retouched), core/choppers, and so on; the latter are associated with much broken bone material. In the Acheulian assemblages cleavers predominate over handaxes and, though these were retouched at the sites where they were used, the primary flakes from which they were made were obtained elsewhere and carried into the activity areas. These flakes appear to have been struck from 'giant polyhedron' cores of basalt that occur as erratics in the Upper Bodo Beds⁶; Wehaietu Formation⁷. Twelve of these cores were studied; each weighed more than 100 kg, averaged ~30 cm on a side and showed the scars

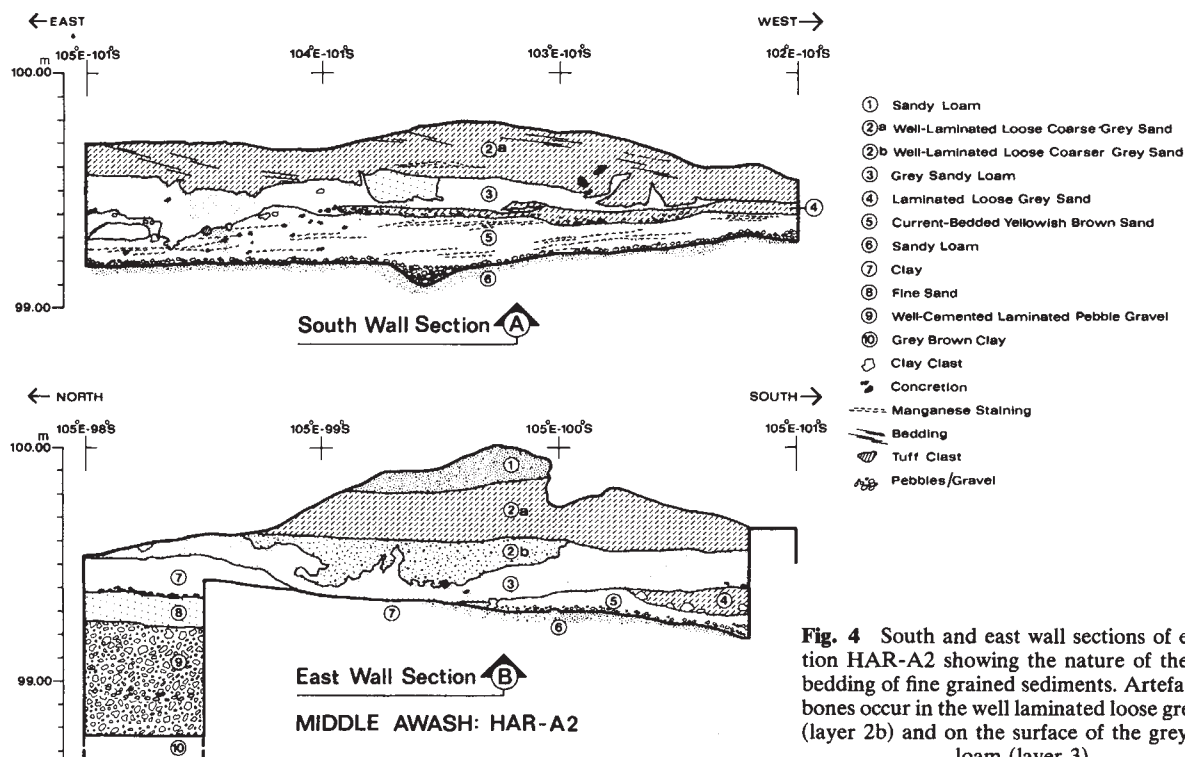


Fig. 4 South and east wall sections of excavation HAR-A2 showing the nature of the interbedding of fine grained sediments. Artefacts and bones occur in the well laminated loose grey sand (layer 2b) and on the surface of the grey sandy loam (layer 3).

of on average from four to six large flakes that had been struck from them.

Assemblages of Developed Oldowan light duty artefacts associated with bone are of two kinds. First, occurrences similar to those from the excavated site BOD-A1 which contains a small concentration (4×3 m) of broken bone representing possible food waste and fresh flakes, all in a fine silty sand. The bone represents fragmentary remains of large, medium and small bovids, a canid, crocodile and catfish. Associated artefacts ($n = 136$) consist of small primary flakes having an irregular planform and mostly plain striking platforms. Also present was a single, radially-flaked biconical core which is likely to have been the source of most of the flakes. Second, occurrences consisting of the remains of a single large animal, usually a hippopotamus, with associated artefacts. These have been considered to be butchery sites⁸. Most of those studied in 1981 were found to be in channel sands and gravels and to represent fortuitous secondary context accumulations of artefacts and bone brought together by fluvial agencies. However, one potential hippo butchery site was partially excavated at Hargufia (HAR-A2, Fig. 3). While the artefacts ($n = 76$) and bone ($n = 38$) are in fresh and unweathered condition and occur within an area of 25 m^2 , they are also in secondary context. The crossbedded and poorly sorted, laminated, grey sand in which they occur (layer 2b; Fig. 4) indicates a relatively low energy current. Hippo bones (including a complete cranium, vertebrae, ribs, an innominate and scapula) show a narrow range of alignment. Joining fragments of a single rib have been displaced by little more than 1 m (Fig. 3) and both heavy and light elements occur together, evidence suggesting that the assemblage had been minimally disturbed before burial. The artefacts represent a low density occurrence and are made mostly from basalt but also from chert, rhyolite and obsidian (Fig. 5, nos 1–6). Shaped tools comprise convex side-scrapers ($n = 2$), polyhedron ($n = 1$) and sub-spheroid ($n = 1$), and there are three modified flake fragments. The remainder consist of unmodified small flakes ($n = 26$), cores ($n = 5$, including two discoid), flake fragments and chunks. Also associated are four lumps of burnt clay. The tight spatial relationship of bones and artefacts makes it likely that this is a butchery site and kit used by middle Pleistocene hominids.

While it is the light duty flake component that has come to be more generally associated with butchery, it should be noted that a single plotted but unexcavated surface scatter (HAR-A4) of 55 handaxes and five cleavers with hippopotamus bone ~1 km north of HAR-A2 may support Jones²⁶ contention that Acheulian bifaces were primarily butchery tools. The absolutely fresh and unabraded condition of the artefacts and bone fragments at HAR-A4 suggests that a large part of a single animal may still remain buried while the uniformity in biface symmetry, size and shape and the absence of trimming flakes suggest that these tools were made and used on a single occasion only.

Larger concentrations of bifaces do exist in the Afar but they appear to be rare and this pattern of small, concentrated occurrences of bifaces and light duty artefacts made for one occasion only is that which we have found to predominate in the area. Moreover, the Acheulian (bifaces) and developed Oldowan (flake/core-chopper) components are usually not found together in the same assemblage, the possibility is suggested that each may represent a different set of cultural activities. Unlike, therefore, the large concentrations of the Ethiopian and south-east plateau sites where both components occur together^{27,28}, these sites in the Afar depression may provide a better opportunity for identifying what the activities may have been.

Fossil hominid discoveries

The 1981 survey resulted in the recovery of the posterior-inferior corner of a large left hominid parietal (BOD-VP-1/1, found by T.D.W.) ~400 m south of the original Bodo hominid discovery⁴ and from the same Middle Pleistocene stratum. The new specimen preserves a part of the vault not seen on the original Bodo find. It represents a second, geologically contemporary adult

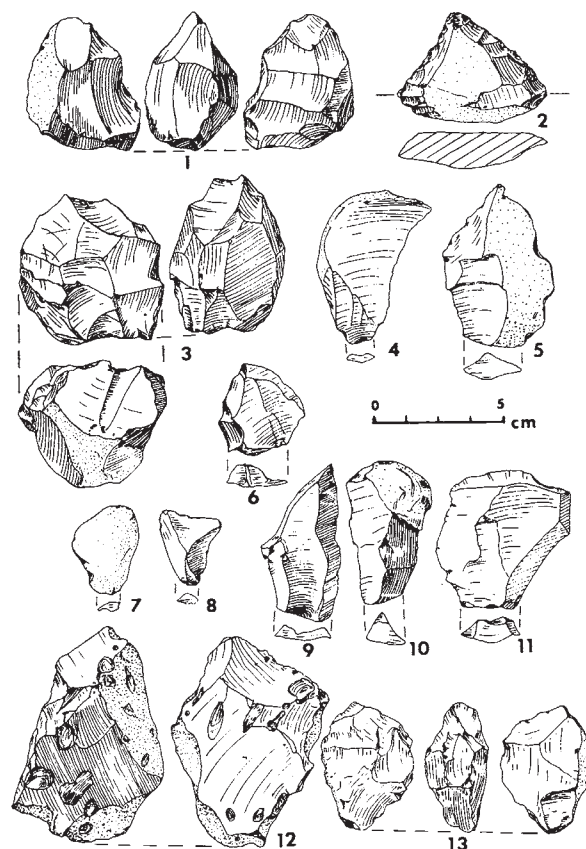


Fig. 5 Nos 1–6: artefacts from the 'Upper Bodo Beds' in the Hargufia drainage. *In situ* in HAR-A2 excavation; possible hippo butchery occurrence. All in fresh condition. 1, Angle core (side chopper): basalt (A2-19). 2, *Dejele* scraper on cortex flake: rhyolite (A2-59). 3, Multi-platformed core (three platforms): basalt (A2-4). 4, Cortex-backed knife on irregular end-struck flake: basalt (A2-20). 5, Irregular end-struck flake with marginal notching: basalt (A2-2). 6, Short sub-triangular flake: basalt (A2-10). Nos 7–13: artefacts from the 'Middle Bodo Beds' in the Bodo drainage. Oldowan Industry artefacts *in situ* from BOD-A3 excavation. All in fresh condition. 7, Irregular end-struck cortex flake: rhyolite. (A3-905). 8, Irregular end-struck flake: basalt (A3-904). 9, Irregular end-struck flake: basalt (A3-907). 10, Irregular end-struck flake (core trimming flake): basalt (A3-880). 11, Irregular end-struck flake: basalt (A3-911). 12, Single platform core (unifacial side and end chopper): vesicular basalt (A3-899). 13, Angle core (bifacial side chopper): basalt (A3-908).

individual of the same taxon. The parietal is robust, measuring 20.8 mm across asterion, the greatest such dimension recorded for a hominid. The specimen features a strong supramastoid crest extension, a massive angular torus and a wide temporal overlap²⁹.

An adolescent left proximal femur of a Pliocene hominid (MAK-VP-1/1) was found at Maka (by T.D.W.) only 7 m above the projected CT horizon in a limonite-stained gravelly sand that yields a diverse and abundant fauna. An erosional disconformity separates the hominid from the radiometrically dated CT horizon. However, the presence of primitive *Notochoerus euilus*, *Nyanzachoerus kanamensis*, *Elephas recki brumpti*, primitive *Theropithecus*, *Ugandax* sp., *Predamalis deturi* and *Ceratotherium praecox* demonstrates that the Maka femoral fragment is at least as old as the Sidi Hakoma member of the Hadar Formation immediately to the north and the Laetoli Beds in Tanzania. The age of the former deposits remains unresolved^{30–32} while the latter are well dated to ~3.7 Myr (ref. 33). We therefore assign an age of 3.5–4.0 Myr to the Maka femoral fragment.

Approximately 700 m north-east of the Maka femur find a second Pliocene hominid fossil was recovered, 11 m below the

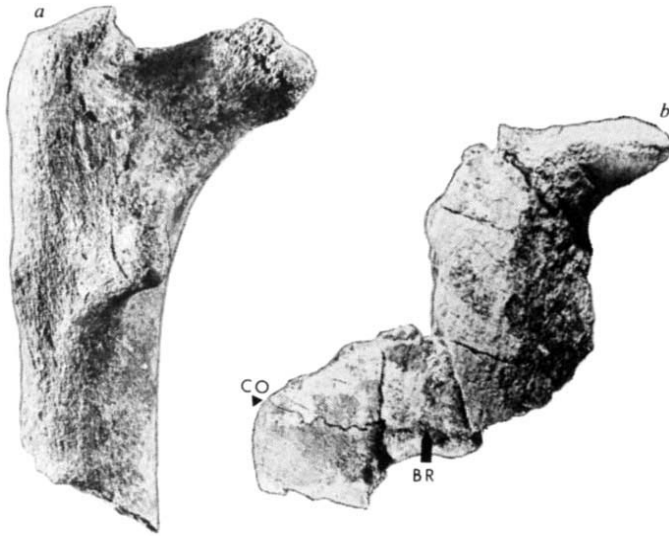


Fig. 6 Pliocene hominids from the Middle Awash, 1981. *a*, Proximal femur from Maka (MAK-VP-1/1). *b*, Frontal from Belohdelie (BEL-VP-1/1). CO indicates the coronal suture and BR indicates bregma. See text for details. See Figs 1 and 2 for geographical and stratigraphical placement. $\times$, 60%.

CT in the Belohdelie drainage. The specimen (BEL-VP-1/1, found by L. Krishtalka) consisted of seven vault fragments eroding from Pliocene sediments. Three of the fragments join to comprise much of an adult hominid frontal. The geological correlation for the two Pliocene hominids with respect to the CT is firm. Matrix on the fossils indicates that these surface finds eroded from the deposits on which they rested. Limited stratigraphical exposure at both localities argues against the fossils' being derived from younger sediments so that these cranial fossils are securely placed at >4.0 Myr, an age consistent with associated fauna.

The new Pliocene fossils from the Middle Awash (Fig. 6) are important because of their antiquity and because they represent functionally critical parts of the hominid skeleton. The Pliocene femur fragment represents an individual whose greater and lesser trochanters had fused. The femoral head was beginning to fuse but is now lost leaving the subepiphyseal surface partially intact. The specimen was virtually adult in size and is intermediate between the smallest (A.L. 288-1, 'Lucy') and the largest (A.L. 333-3) Hadar femora. It agrees in detail with the anatomy seen in *A. afarensis*³⁴. A suite of external morphological features diagnostic of habitual bipedality is evident on the Maka specimen. These characters include the position of the lesser trochan-

ter, the length of the neck, the morphology of the obturator insertion, the shape of the superior notch, the height of the greater trochanter and the clear obturator externus groove. The specimen is susceptible to a variety of X-ray techniques, including xeradiography and computerized tomography. The presence of thickened cortex through the ventral portion of the neck and the strong calcar also indicate habitual bipedality.

The Belohdelie specimen is stratigraphically older than the Maka femur. This fossil is of outstanding importance because it fills the last unknown area of adult cranial anatomy in the earliest Pliocene hominids. The only other hominid vault remains approaching this age belong to *A. afarensis*³⁵. The Belohdelie frontal resembles that of the Laetoli child (L.H.-21)³⁵ and conforms well to predictions made by a composite cranial reconstruction of an adult Hadar male³⁶. The new fossil is thick, measuring 8.4 at 10.0 mm anterior to bregma. Frontal length is diminutive and the overall size of the bone is equivalent to that of an adult chimpanzee. The small size and apelike morphology of the supraorbital corner combine to indicate a primitive status for the fossil. The lack of marked postorbital constriction or strong temporal line convergence is different from the robust *Australopithecus* condition. The small vault size and long, low, flat frontal squama set the specimen apart from *Homo*. Vault thickness and lack of a supratrochlear sulcus are traits not seen in African pongids. The morphology of BEL-VP-1/1 suggests that the fossil is best considered to belong to *Australopithecus*, a hominid genus. The specimen constitutes a significant addition to our knowledge of Pliocene hominid form and emphasizes the primitive nature of early hominid cranial architecture.

We anticipate that further fieldwork in this area will yield additional significant results.

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Origin and evolution of the Mediterranean vegetation and climate in Europe

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Palynology provides information about Pliocene and early Pleistocene vegetation and climate evolution in the north-west Mediterranean area in relation to high northern latitude climatic trends. An important change occurred approximately 3.2 Myr ago with the appearance of the Mediterranean climatic rhythm (summer drought) causing the individualization of the modern Mediterranean floral elements. Quaternary-type mediterranean climatic fluctuations started approximately 2.3 Myr ago (early Glacial) causing the Mediterranean-type of vegetational organization.

MEDITERRANEAN climatic conditions are today distinctive and consist of a temperate climate characterized by dry summers with rainfall concentrated during the other seasons and low temperatures during winter. Floral assemblages and the vegetational organization are therefore very typical. To understand present day ecosystems it is essential to establish when and how the summer drought first occurred.

A model based on fossil macrofloras was proposed for California¹ (a region now displaying the same climatic and vegetational features as the Mediterranean area^{2,3}) but there is at present no equivalent for the Mediterranean area where recent advances in palaeoclimatic knowledge are based mainly on studies of marine deposits, particularly from DSDP site 132, according to foraminifera^{4,5}, isotopic measurements⁶ and clay minerals⁷. This research has yielded information about water temperatures and about rainfall rhythm. Until recently, reliable palaeoenvironmental floristic records based on pollen grains⁸⁻¹⁰ and macroremains^{11,12} were scarce.

Our own palynological research has been concentrated in the Gulf of Lion (off-shore sequences and terrestrial sections in Languedoc, Roussillon and Catalunya). New data have been obtained from off-shore sections in the Adriatic Sea and from lacustrine deposits in southern Italy (Fig. 1). Long palynological sequences covering the whole Pliocene and early Pleistocene were yielded by off-shore sections (Autan 1¹³ and Garraf 1¹⁴ in the Gulf of Lion and the Adriatic Sea, J. Cravatte and J.-P.S., unpublished data) in which the biostratigraphic control is based on planktonic foraminifera and nannoplankton. The foraminiferal zonation adopted in this article has been determined in the Gulf of Lion¹⁵ with reference to the DSDP site 132 chronology¹⁶: *Globorotalia margaritae* s.l. zone, the top of which is marked by *G. puncticulata* decreasing or absent just above the *G. margaritae* extinction (from approximately 5.4 to 3.2 Myr ago); *G. crassaformis* zone, the top of which is marked by the first appearance of *G. inflata* (3.2–2.2 Myr ago); *G. inflata* zone, the top of which is marked by the first appearance of *Hyalinea balthica* (2.2–1.8 Myr ago). Short terrestrial sections (marine coastal or lacustrine deposits) have been investigated^{14,17-19} and their chronological placement obtained from mammal biochronology²⁰ and sometimes from K–Ar (ref. 21) and palaeomagnetic measurements (refs 22, 23 and N. A. Möerner, personal communication).

Appearance of Mediterranean climate

During the Lower Pliocene (Tabianian stage, approximately represented by the *G. margaritae* s.l. zone¹⁵), the north-west Mediterranean area had a dense forest vegetation composed of: (1) a coastal association in which Taxodiaceae predominated (*Taxodium*-type especially), *Myrica*, *Symplocos*, *Nyssa*; and (2) just behind it, assemblages with *Engelhardtia*, *Carya*, *Rhoiptelea*, *Hamamelis*, *Embolanthera* growing in less damp biotopes.

Moist climatic conditions (with rainy summers) prevailed during

this time (P I pollen zone²⁴; Fig. 2A) and climatic fluctuations are revealed by pollen assemblages of the subzone P Ib, denoting less humid conditions. Such assemblages can be compared with the present evergreen sclerophyllous broad-leaved forest in China²⁵.

A great change occurred approximately 3.2 Myr ago that modified the composition and structure of the forests. The coastal forest swiftly grew thinner and disappeared. Components of the sclerophyllous forest also changed and *Quercus* species and *Alnus* (in damp biotopes) became predominant. Furthermore, xerophytic taxa (those of the present-day Mediterranean) rose by degrees to higher frequencies (*Phillyrea*, *Olea*, *Cistus*, *Quercus ilex*-type, *Pistacia*). During the beginning of this phase all species of Taxodiaceae disappeared from Languedoc-Roussillon²⁶.

The P I–P II boundary (Fig. 2A) is a climatic event, rather than related to the local Piacenzian transgressive phase, not only because of the disappearance of the coastal forest which does not occur everywhere (in Catalunya, it diminished but did not disappear at that time), but also because of the change in the structure and the composition of the sclerophyllous forest. Moreover, this boundary precedes (Rhône Valley) or follows (Catalunya) the transgressive movement. During the same time, some subtropical planktonic foraminifera disappeared from the Mediterranean Sea (*G. margaritae*, *G. puncticulata* and *Sphaeroidinellopsis*⁴).

Vegetational assemblages were certainly complex. Although a latitudinal and altitudinal zonation existed, a mosaic distribution most likely existed in relation to the relief, and different types of soils: for instance, *Cedrus* pollen grains are only abundant in western Languedoc and less so in Catalunya deposits which are located near south-facing metamorphic and granitic mountains; *Abies*, *Picea* and *Fagus* pollen grains are numerous at the opening of the Rhône Valley.

At the beginning of the P II pollen zone (Fig. 2A), all taxa requiring a year-long degree of moisture decreased or disappeared while xerophytic ones increased (frequencies and absolute counts²⁴). *Phillyrea*, *Olea*, *Cistus*, *Quercus ilex*, *Pistacia*, which ultimately came to characterize the Mediterranean flora, were already abundant and appear to have been the most resistant to the new climatic conditions and the most adaptative but did not yet form specialized associations. Today, similar assemblages are developed in the eastern Mediterranean (Pontic) area where *Phillyrea media*, *Quercus ilex*, *Rhamnus alaternus* grow under humid conditions (maximum rainfall in summer) and are associated with deciduous forest (*Quercus*, *Pterocarya*, *Fagus orientalis*)²⁷. *Pistacia*, *Phillyrea*, *Cistus*, *Olea* and the *Quercus ilex*-group (evergreen species) were present in the north-west Mediterranean area at the base of the Miocene beds (sometimes in high percentages) and mixed with tropical assemblages²⁸.

Also during P II, sedimentary trends evolved into coarse and red deposits chiefly in Languedoc-Roussillon. The significance

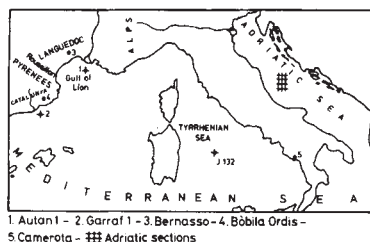


Fig. 1 Location map of the Plio-Pleistocene palynological sequences utilized in this paper. The planktonic foraminifera biostratigraphy of Autan 1 and Garraf 1 wells is referred to the DSDP site 132 chronostratigraphy.

of this has been explained as a shrinking of the forest surface caused by an increase in xericity²⁹. The composition of the Ruscinian mammal faunas which mostly correlate with the P II pollen zone is in accordance with the new vegetational and climatic characteristics of that time. For instance, the Muridae, which predominated among rodents, are now living in open forest subtropical biotopes³⁰.

According to all these recent data, we consider that at the beginning of the Piacenzian stage (approximately 3.2 Myr ago) the climate of the north-west Mediterranean area evolved towards a seasonal rhythm of great contrast and the summer drought became stable approximately 2.8 Myr ago (Fig. 3). This opinion is supported by the changes in vegetation occurring later and hereunder described.

Earliest modern-type climatic fluctuations

In the upper part of the *G. crassaformis* zone which overlaps with the first appearance in the Mediterranean Sea of the planktonic foraminifera *G. inflata* (until then characteristic of northern latitudes)¹⁵, 'steppic' pollen assemblages were on the increase (*Artemisia*, *Ephedra*, other Compositae, Caryophyllaceae, Amaranthaceae-Chenopodiaceae, *Erodium*)³⁰ compared with low frequencies of arboreal pollen grains (*Pinus* excepted). The P III pollen zone, as defined in the Autan 1 and Garraf 1 sections, has a climatic significance without any relation to the marine regression in the Gulf of Lion which occurred much earlier (Fig. 2A)²⁴. Moreover, this climatic zone, which lasted about 0.2 Myr (approximately from 2.3 to 2.1 Myr ago³¹), was also registered in the Adriatic series (J. Cravatte and J.-P.S., unpublished data). Pollen spectra clearly demonstrate a reduction of forest cover and the enlargement of 'steppic' vegetational associations denoting drier and probably milder climatic conditions²⁴ (Fig. 3).

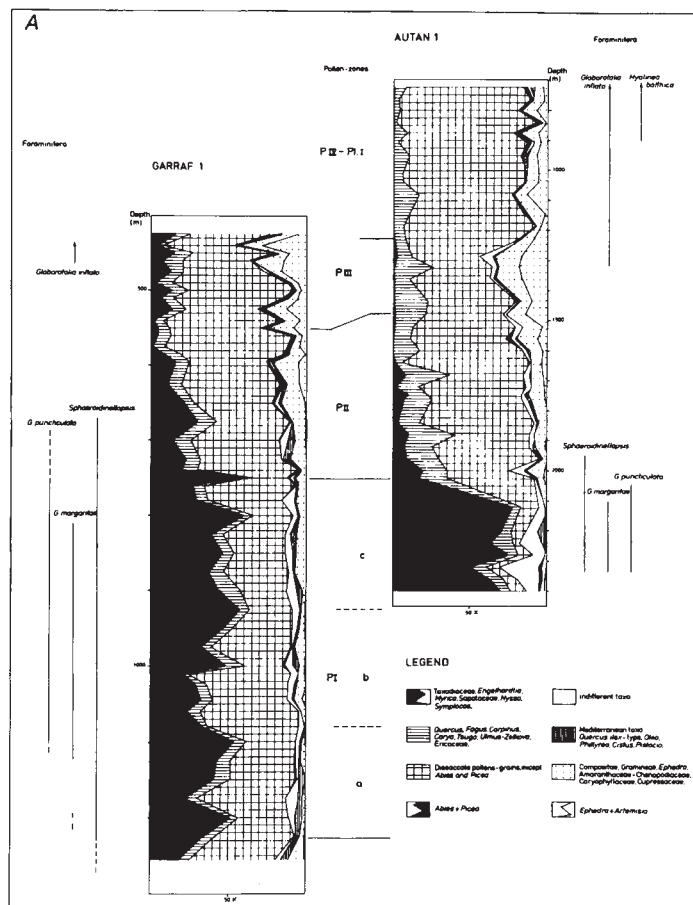
The next pollen zone (P VI-Pl. I) where the Pliocene-Pleistocene boundary occurs was recognized in southern Italy¹⁹ (Camerota) and in the Adriatic sections. Forest associations predominated again but *Quercus*, *Carya*, *Carpinus*, *Ulmus-Zelkova* were then the most abundant taxa along with *Acer*, *Pterocarya*, *Parrotia persica*. Palaeogeographic reconstructions and comparisons between pollen- and macro-floras show an established Mediterranean vegetational zonation (P. Brenac, personal communication) as follows:

(1) The coastal *Olea-Ceratonia* association (accompanied by *Pistacia*, *Phillyrea* and *Myrtus* that is, thermo-Mediterranean).
(2) The *Phillyrea-Quercus ilex* association (accompanied by *Olea*, *Carpinus orientalis* and *Rhamnus*; that is, meso-Mediterranean).

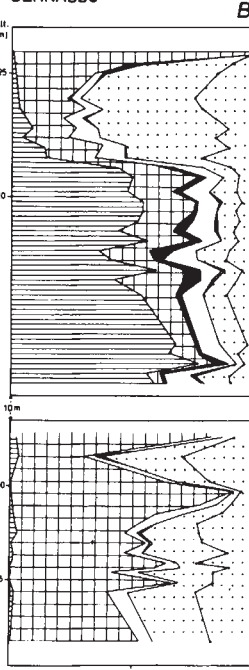
(3) The deciduous *Quercus* forest with *Carpinus* (*C. orientalis* and *C. betulus*), *Carya*, *Ulmus* and *Zelkova* (the supra- or humid-Mediterranean zone).

(4) Mountain forest including *Cedrus*, *Pinus*, *Abies*, *Picea* and *Tsuga*.

This is an altitudinal zonation characterized by increase in moisture and decrease in temperature. The present model of such forests must be sought in Pontic and to a less degree in Caspian areas^{27,32}. From a climatic point of view, the P VI-Pl. I phase



BERNASSO



BÒBILA ORDIS

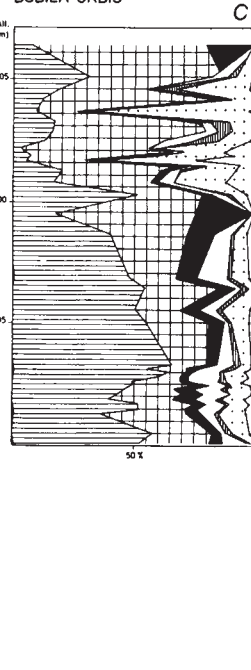


Fig. 2 Synthetic pollen diagrams based on ecological assemblages. A, Garraf 1 and Autan 1 with the planktonic foraminifera first and last appearance data; the pollen zones are founded on the vegetational changes; notice the decrease of taxa requiring a year long degree of moisture (P I-P II boundary) and the increase of 'steppic' elements (P III pollen zone). B, Bernasso: two 'steppic' phases are bordering a deciduous forest period (interstadial). C, Bòbila Ordis: an interglacial deciduous forest phase.

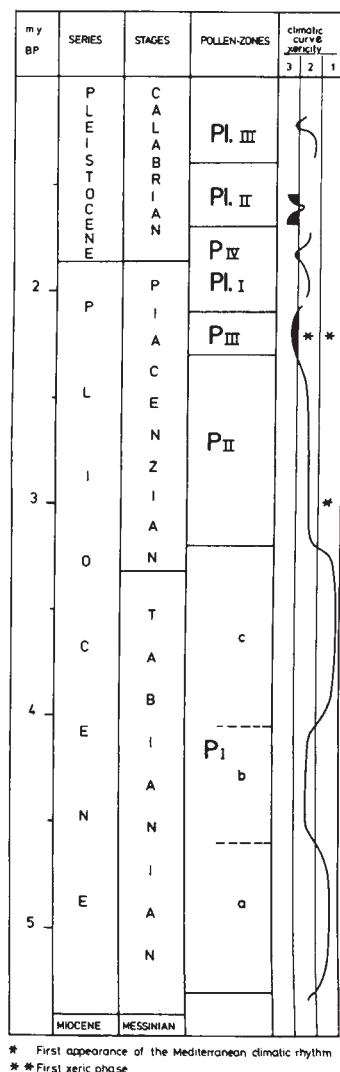


Fig. 3 Climatic evolution in north-west Mediterranean area during the Pliocene and Lower Pleistocene. The Quaternary-type climatic fluctuations began with the oldest 'steppic' phase P III.

indicates a return to moister (rainfall increasing) and warmer conditions²⁴ (Fig. 3).

With the P III period, the climate started fluctuating as it did in the late Pleistocene. The oldest cycle is constituted by P III and P IV–Pl. I phases, the second one²⁴ (Fig. 3) includes Pl. II (recognized at Bernasso¹⁸ and in Adriatic sections—Fig. 2B; drier and probably cooler than P III) and Pl. III phases (recognized at Bòbila Ordis³³ and Nogaret—Fig. 2C; certainly warmer than P IV–Pl. I); the next cycle is incompletely known up till the lowermost oscillation in the Tenaghi Philippon pollen diagram (northern Greece) which is dated at 0.7 Myr ago³⁴.

These mean early climatic periods can be subdivided into several minor phases (Figs 2B, 2C, 3). The abrupt transition between two alternating minor phases appears difficult to understand but the possibility of an intermediate climate oscillating over both sides of a threshold should be considered. This is one of the reasons why we do not consider that early 'steppic' phases denote cool conditions but only drier and perhaps milder ones. Another argument is provided by some differences between the oldest and most recent 'steppic' associations as is indicated in the early Pleistocene by the presence of thermophilous elements (*Cistus*, *Phlomis* cf. *fruticosa*, *Erodium*). Thus it would be possible to discriminate between ancient Mediterranean 'steppes' and recent ones by reference to the differentiation now existing between steppes with dry and cool determinism²⁷. This supports the idea of dividing the Mediterranean Pleistocene into two distinct climatic periods, the earliest warmer and the latest

characterized by important temperature fluctuations^{5,35}. On the other hand, the transition between two main successive vegetational (then climatic) phases appears to have been gradual as proved by the different noticeable subdivisions in the P VI–Pl. I pollen zone. In short, the modern type of Mediterranean climatic fluctuations started in the late Pliocene; the periodicity and the amplitude of these successively dry and humid periods increased up to the present.

Conclusions

Chronological relationships between the Mediterranean Pliocene–Pleistocene climatic fluctuations and those defined in north-west Europe^{36,37} can be established. Correlations are based both on independent chronological data and on the correspondence between Mediterranean 'steppic' and tundra-like pollen spectra on the one hand and forest pollen assemblages on the other corresponding respectively to late Glacial and Holocene models³¹. Before the P III pollen zone, relationships between the Mediterranean area and north-west Europe are less obvious than in later periods; nevertheless an equal number of climatically connectable changes is observed in the two areas³¹.

The following relationships³¹ are thus proposed (Fig. 3):

Pl. III	Waalian	P III	Praetigian
Pl. II	Eburonian	P II	Reuverian
P VI–Pl. I	Tigian	P I	Brunssumian

Therefore, climatic changes which occurred in the Mediterranean area are essentially related to temperature changes in the high northern latitudes³¹.

The cooling dated at approximately 3.2 Myr ago was a global event that has been recognized in planktonic foraminifera^{4,5,38} and oxygen isotopic curves^{6,39,40}. In the Mediterranean area, the same trend in isotopic curve could indicate "an increased evaporation relative to precipitation"⁶ which would be in accordance with information deduced from pollen records. The P I–P II boundary climatic event is probably related to this cooling caused by the beginning of the permanent glaciation in the Arctic area which in turn is related to the Gulf Stream intensification due to gradual uplift of the Panama isthmus⁴¹. This climatic event was preceded by the less humid P Ib phase in the Mediterranean area (that is, Brunssumian B in north-west Europe³¹) as also indicated by planktonic foraminifera data⁵. The climatic trends as revealed by palynological records in the Mediterranean area are quite similar to those indicated by data obtained from planktonic foraminifera and isotopic studies. Furthermore, palynology yields additional information about changes in moisture. The two main climatic changes occurred at around 3.2 and 2.3 Myr ago, the greater importance of the later event being generally accepted today (L. D. Keigwin, Jr. personal communication). Moreover, according to these different methods there is wide agreement that the Pliocene–Pleistocene boundary is not characterized by climatic cooling^{4,5,24,31}.

The pollen analysis allows distinction in the Mediterranean area between interglacial and interstadial forest phases. For instance, the forest phase in the Bòbila Ordis pollen diagram corresponds to an interglacial period shown by high frequencies of deciduous oaks (that is, the interglacial forest climax in the Mediterranean area), the presence of the two hornbeam species (*Carpinus orientalis* and *C. betulus*) and *Parrotia persica* as opposed to low percentages of Mediterranean 'steppic' elements even in the upper part of the pollen diagram (Fig. 2C); moreover, the relative high representation of Mediterranean taxa and the succession in vegetational features are characteristic. In contrast, very low frequencies of deciduous oaks and only one hornbeam species (*C. orientalis*) characterize the interstadial forest phase in Bernasso pollen diagram while Mediterranean 'steppic' components reach high values (Fig. 2B).

The beginning of the evolution of the Mediterranean-type climate has been characterized more by fluctuations in moisture (especially in rainfall rhythm) than in temperature. From the early Pliocene onwards, the climate was already fluctuating but the period and the amplitude increased during the Pleistocene.

Two very important steps are located at approximately 3.2 Myr ago (progressive establishment of the Mediterranean rhythm with dry summers; individualization of the modern Mediterranean floral elements) and 2.3 Myr ago (the oldest xeric phase; starting of the Quaternary-type oscillations; oldest signs of a Mediterranean-type vegetational organization). The previous flora was decimated and the vegetation thoroughly modified

because of the intensity and rapidity of the climatic changes and, finally, human activity had a considerable effect.

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Construction, expression and recognition of an H-2 molecule lacking its carboxyl terminus

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A mouse major histocompatibility antigen (H-2) gene, encoding a novel H-2L^d molecule lacking its intracytoplasmic domain, has been constructed and introduced into mouse L-cells. The novel H-2 molecule is found on the surface of the transfected cells at the same level as L-cells transfected with the native H-2L^d gene. Allo- and influenza-specific cytotoxic T lymphocytes can recognize the truncated H-2 gene product nearly as efficiently as the normal H-2L^d gene product. However, vesicular stomatitis virus-specific cytotoxic T lymphocytes recognize the truncated H-2L^d molecule less efficiently than the complete H-2L^d product. The rate of capping of the truncated H-2L^d molecule was investigated and found to be the same as that of the complete H-2L^d gene product.

THE major histocompatibility complex (MHC) of the mouse encodes a variety of cell-surface glycoproteins involved in the immune response^{1,2}. H-2K, H-2D and H-2L are the three murine major histocompatibility antigens that have been defined by classical genetic and serologic criteria. These transplantation antigens are members of a family of proteins referred to as class I antigens. The class I molecules are glycoproteins that consist of two subunits, β_2 -microglobulin and H-2. The 12,000 molecular weight β_2 -microglobulin chain is found only on the extracellular part of the protein. The H-2 subunit is about 345 amino acids long and is encoded by a gene comprised of 8 exons^{3–5} (Fig. 1). The first 280 residues are on the extracellular side of the cell membrane, 40 residues are thought to be imbedded in the cell membrane and the final 25 residues are inside the cell. The role of these intracellular residues of the H-2 molecule is the subject of this article.

Class I molecules serve as targets for T-cell mediated cytotoxicity in allogeneic immune responses, such as *in vivo* graft rejection and destruction of allogeneic cells by cytotoxic T lymphocytes (CTLs) *in vitro*. However, the physiological role of transplantation antigens might be to serve as a restriction element in T-cell

lysis of virus-infected cells. That is, virus infection of mice generates CTLs whose receptor(s) must interact with the foreign viral antigens and a class I molecule in order to lyse the virus-infected cell^{6,7}.

Genomic clones encoding H-2L^d and H-2D^d can be introduced into thymidine-kinase deficient mouse L-cells by co-transformation with the herpes thymidine kinase gene^{8,9}. The gene products are functionally expressed and can mediate immunologically specific cellular interactions^{10,11}. Recently, we have constructed H-2D^d/H-2L^d hybrid genes expressing the N and C₁ domains of one molecule and the C₂, M and cytoplasmic domains of the other¹². The restricting elements for allo- and antiviral CTLs were mapped to the N and/or C₁ domains^{13,14}. This approach allows us to begin to define the portions of these molecules important for CTL recognition.

In the present studies, we sought to determine whether the cytoplasmic portion of the H-2 molecule has a role in the process by which T cells lyse their targets. A variety of functions can be postulated for the intracytoplasmic portion of the H-2 molecule. To test these models, we constructed an H-2L^d gene in which the cytoplasmic domains have been deleted. H-2 anti-

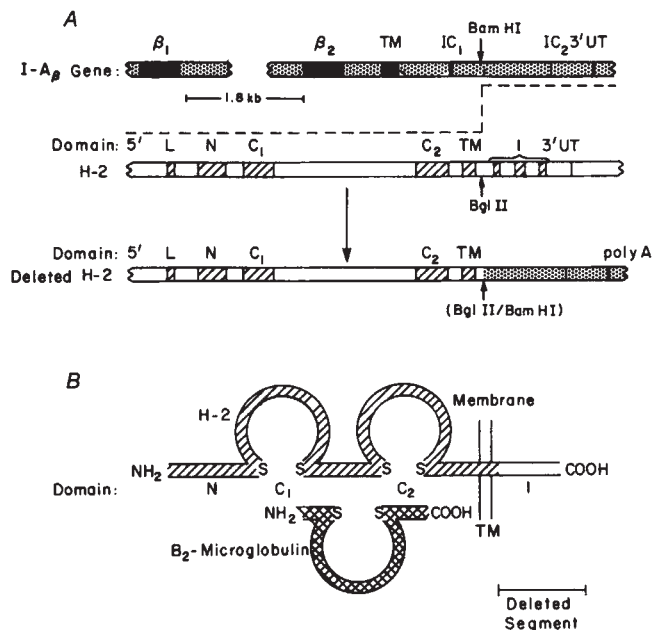


Fig. 1 Construction of the truncated *H-2L^d* gene (A) and the predicted structure of the deleted H-2 antigen (B). The truncated *H-2L^d* gene was made from restriction enzyme fragments using standard recombinant DNA technology³². A fragment encoding a deleted gene was isolated after a partial digestion of pL^d-4 plasmid DNA with *Bgl*II. A 2.5-kb fragment encoding the last cytoplasmic domain of the murine *I-A_β* gene cloned into the *Bam*HI site of pBR322 was provided by Dr E. Choi. Partial digestion of this plasmid DNA with *Bam*HI yielded a single fragment containing the 2.5 kb *Bam*HI fragment and the pBR322 sequence. The *Bgl*II fragment encoding the deleted *H-2L^d* gene was ligated to the pBR322-*I-A_β* fragment, and used to transform *Escherichia coli* strain LE322³³. The *Bgl*II and *Bam*HI sites destroyed by this procedure are indicated as (*Bgl*II/*Bam*HI) in the deleted gene (A). Colonies were screened for the insert having the correct orientation by a 'mini-DNA' preparation followed by restriction enzyme digestion and agarose gel electrophoresis³⁴. The resulting plasmid was extensively mapped by restriction-endonuclease cleavage to confirm the reconstruction and that no other rearrangements of the coding regions had occurred. Black areas represent the region coding for the *I-A_β* gene¹⁶. Hatched areas represent the regions coding for the *H-2L^d* gene^{4,5}. Stippled areas represent the intervening sequences and 3' untranslated region of the *I-A_β* gene. β_1 , β_2 , TM, IC₁, IC₂, L, N, C₁ and C₂ represent the coding domains of the *I-A_β* and *H-2L^d* genes. The portion of the H-2 molecule that should be deleted by this procedure is indicated in the lower panel.

Table 1 *H-2L^d* antigens on the surface of L-cells containing native and deleted *H-2L^d* genes

Cell	<i>H-2L^d</i> gene	¹²⁵ I-Protein A bound
L cell	—	700
T1.1.1	Native	25,620
T123.8	Native	14,620
D3.8	Deleted	24,642
D3.4	Deleted	19,337

Cloned *H-2* genes encoding either the native or deleted *H-2L^d* gene were introduced into thymidine kinase deficient mouse L-cells (DAP-3) by DNA-mediated gene transfer using the herpes simplex thymidine kinase gene as a selectable marker as described previously^{4,8}. The amount of *H-2L^d* expressed on the cell surface was measured by radioimmunoassay^{8,12}. 2×10^5 cells were incubated in 50 μ l of a 1:1 dilution of a monoclonal *H-2L^d* antibody (28.8.14) for 2 h at 4 °C. The cells were extensively washed, ¹²⁵I-labelled Protein A was added, and the cells were incubated for an additional 2 h at 4 °C. After extensive washing the amount of ¹²⁵I bound to the cells was measured. Each value represents the mean of triplicate samples with a standard deviation of less than 3% of the mean. Although the relative amounts of *H-2L^d* on the surface varied somewhat from experiment to experiment, the amount of *H-2L^d* on the surface of T1.1.1 was always very similar to the amount on the surface of the D3.8 cells, while the T123.8 and D3.4 cells had about 50% and 80% respectively of the *H-2L^d* found on the surface of T1.1.1.

restriction nuclease site in the intron between the membrane and first cytoplasmic domain^{4,5}. Specifically, four *Bgl*II sites are present in the plasmid pL^d-4 (not shown in Fig. 1). Two *Bgl*II sites are located 5' to the promoter region. One *Bgl*II site is located in the C₂ domain and one in the intervening sequence between the membrane and first cytoplasmic domain. Plasmid pL^d-4 DNA was partially digested with *Bgl*II and a fragment was isolated encoding the *H-2L^d* molecule without the cytoplasmic portion. Recently, the nucleotide sequence of the coding portions of the mouse *I-A_β* gene has been determined¹⁶. A 2.5-kilobase (kb) *Bam*HI fragment from the 3' end of the *I-A_β*^k gene encodes a 3' acceptor site, three amino acids, a terminator, a 3'-untranslated region and a poly(A) addition site (Fig. 1A). The *Bgl*II fragment encoding the *H-2L^d* molecule, without the cytoplasmic portion, was ligated to the 2.5 kb *Bam*HI fragment. This construction resulted in an *H-2L^d* gene in which the *H-2* cytoplasmic domains were deleted (Fig. 1).

Figure 2 shows the amino acid sequence of the transmembrane region and the 25 amino acids comprising the cytoplasmic domain of the native *H-2L^d* protein. We expected the constructed *H-2L^d* gene to encode an *H-2L^d* molecule with the same transmembrane region as the native *H-2L^d* molecule, but lacking its cytoplasmic portion. The IC₁, IC₂ and IC₃ encoded portions of the native *H-2L^d* molecule should be substituted by three amino acids encoded by the cytoplasmic domain from the *I-A_β*^k gene (Fig. 2).

Deleted *H-2* gene products in transformants

To establish whether the truncated gene is expressed when reintroduced into mouse cells, DNA from either the truncated or the complete *H-2L^d* gene together with the herpes viral thymidine kinase gene, was used to co-transform thymidine kinase negative mouse L-cells as previously described^{17,18}. Transformants were selected in hypoxanthine-aminopterin-thymidine medium and screened with monoclonal antibodies specific for *H-2L^d* transformants^{19,20}. Cell-surface antigen expression was measured quantitatively by radioimmunoassay. Two cloned lines transformed with the deleted *H-2L^d* gene, D3.4 and D3.8, were chosen for further analysis. Table 1 shows that clone D3.8 had as much *H-2L^d* on its surface as was found on the surface of clone T1.1.1 (an L-cell transformed with the complete *H-2L^d* gene, as described previously³). Clone D3.4, used in the biochemical studies described below, had slightly less *H-2L^d* molecules on its surface. Apparently, the deleted *H-2L^d* expressed after introduction into L-cells can be recognized by monoclonal antibodies.

To demonstrate that the deleted gene product was associated with β_2 -microglobulin and shorter than the native protein, the deleted protein was isolated from the cell surface. Membrane

gens, like other cell-surface molecules, 'cap'. Several models to explain the formation of these caps have been proposed¹⁵. We have compared the capping of the native and deleted H-2 molecules in order to understand the role of the intracytoplasmic region of the molecule in the capping process.

H-2 molecules with cytoplasmic part deleted

A variety of approaches are available for removing the segment of a gene encoding the COOH end of the protein. For example, Rose and his collaborators have deleted the 3' end of a cloned cDNA of the vesicular stomatitis virus (VSV) G protein by exonuclease digestion (J. Rose, personal communication). The one constraint imposed on restructuring the gene at the 3' end appears to be that a poly (A) addition site must be encoded at the 3' end of the gene. The approach we have taken involves replacement of the exons that encode the intracytoplasmic portion of the H-2 molecule with the portion of the *I-A_β*^d gene that encodes three amino acids and a poly (A) addition site. One advantage of this approach is that the mRNA from the mutant gene is significantly different from the original H-2 gene in that it hybridizes to *I-A_β* probe.

Detailed structural analysis of the *H-2L^d* gene including the determination of the complete DNA sequence, revealed a

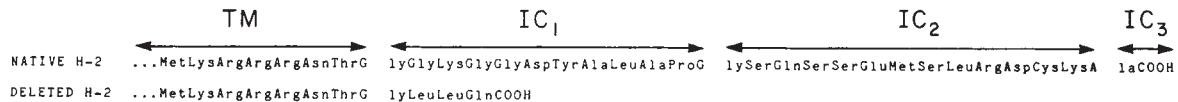


Fig. 2 Amino acid sequence of the transmembrane and cytoplasmic domains of the native and deleted $H-2L^d$ molecules. The amino acid sequence of the transmembrane and cytoplasmic domains of the complete $H-2L^d$ gene were predicted from DNA sequence analysis^{4,5}. The four amino acids encoding the cytoplasmic domain in the truncated $H-2L^d$ gene were derived from the second cytoplasmic domain of the murine $I-A\beta$ gene¹⁶.

Fig. 3 SDS-polyacrylamide gel electrophoresis analysis of ^{125}I -labelled $H-2L^d$ antigens immunoprecipitated from clone T1.1.1, expressing the complete $H-2L^d$ gene, and clone D3.4 expressing the truncated $H-2L^d$ gene. Cell-surface iodination with the ^{125}I was catalysed by lactoperoxidase³⁵. The radiolabelled cells were lysed with 1% Nonidet P40 in 0.01 M Tris-HCl, pH 7.0, 0.15 M NaCl, 1 mM phenylmethylsulphonyl fluoride, 0.02 mg ml⁻¹ ovomucoid trypsin inhibitor. Nuclear debris was removed from the lysates by centrifugation at 13,000g for 15 min at 4 °C. Further pretreatment of the lysates was done by centrifugation at 100,000g for 30 min and by pre-clearing with formalin-fixed *Staphylococcus aureus* bacteria (strain Cowan I) and with a preformed complex of mouse IgG and rabbit-anti-mouse IgG as described previously³⁶. Pre-cleared lysates were incubated for 3–4 h with a preformed complex with the monoclonal 30.5.7 and rabbit anti-mouse IgG at 4 °C and immunoprecipitates were removed from the lysates by centrifugation at 13,000g. Precipitates were resuspended in 0.2 ml Tris-HCl buffer with 0.5% deoxycholate sodium salt and spun through on a discontinuous gradient consisting of 0.4 ml of 10% sucrose, 0.5% Nonidet P-40 and 0.8 ml of 20% sucrose. SDS-polyacrylamide electrophoresis was carried out on discontinuous vertical slab gels according to a modification of the Laemmli procedure³⁷. A gradient gel was made in 10–15% acrylamide. The gels were dried and exposed for 3 days. The figure shows $H-2L^d$ antigens from clone T1.1.1 expressing the complete $H-2L^d$ gene and clone D3.4 expressing the truncated $H-2L^d$ gene.

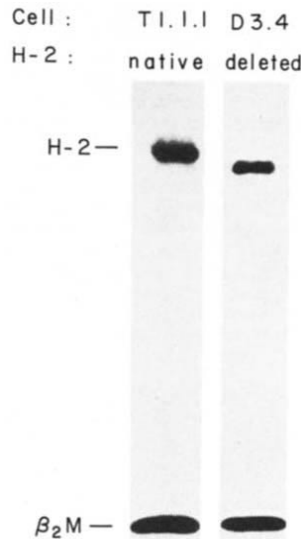


Table 2 Normalized T-cell mediated lympholysis of normal and deleted $H-2L^d$ genes expressed on L-cells

Target	BALB/c anti-VSV BALB/c %	n	BALB/c anti-PR8 BALB/c %	n	C-H-2 ^{dm2} anti-BALB/c %	n	BALB/c anti-C3H %	n
T1.1.1	100		100		100		100	
T123.8	84 ± 10	(5)	81 ± 22	(4)	106 ± 19	(8)	87 ± 7	(9)
D3.4	20 ± 5	(7)	74 ± 17	(5)	72 ± 11	(15)	83 ± 12	(9)
D3.8	19 ± 7	(5)	90 ± 4	(4)	92 ± 16	(8)	74 ± 12	(9)

BALB/c mice were immunized intraperitoneally with either wild-type VSV (10^7 plaque-forming units (PFU)) or influenza A/PR/8/34 virus (100 haemagglutinating units). One week later, the mice were killed and their spleens used as a source of virus-immune lymphocytes. Five-day secondary *in vitro* cultures were established with 5×10^6 primed lymphocytes and 1×10^6 irradiated BALB/c splenocytes which were infected with either 10 PFU tsG41 VSV or 10 EID₅₀ (50% egg infectious dose) PR8 virus per cell in 17 mm Costar dishes with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. Primary allo-specific CTLs were generated in 5-day cultures of 5×10^6 naive lymphocytes derived from either BALB/c or C-H-2^{dm2} mice cultured with 1×10^6 irradiated C3H or BALB/c stimulator cells using the same medium. Virus-specific or allo-specific effectors were collected and dispensed in duplicate at 1×10^6 , 2×10^5 and 5×10^4 cells per V-bottom microtitre well (Linbro) in 0.1 ml minimum essential medium supplemented with 10% FBS, nonessential amino acids, penicillin, streptomycin, and L-glutamine. L-cell targets, detached by versene treatment, were divided into three aliquots and infected with either 10 PFU wild-type VSV or 10 EID₅₀ PR8 virus, or not infected. Target cells were pulsed with 0.1 mCi Na₂⁵¹CrO₄ for 1 h at 37 °C, incubated an additional 1 h, and then washed three times. L-cell targets were dispensed at 1×10^4 cells in 0.05-ml volumes into wells containing effectors, normal spleen cells to measure spontaneous release or 0.1 ml 5% Triton X-100 (to measure total releasable ⁵¹Cr). Plates were centrifuged at 100g and then incubated at 37 °C for 5 h. Supernatants (0.1 ml per well) were collected and counted for ⁵¹Cr release. Per cent specific release was calculated by the formula = $100 \times (\text{experimental c.p.m.} - \text{control c.p.m.}) / (\text{total c.p.m.} - \text{control c.p.m.})$ ¹⁵. In order to present the results from multiple experiments, data were normalized such that lysis of T1.1.1 cells was 100%. Lysis of the T1.1.1 cells was equal to or greater than 70% in all experiments. Standard deviation and number of experiments (in parentheses) are indicated. Data are presented for lysis on appropriate targets (for example PR8 CTLs assayed on PR8 infected targets). Lysis on uninfected targets was always less than 10%.

proteins were labelled by lactoperoxidase-mediated iodination of the transformed cells. The proteins were immunoprecipitated by monoclonal antibody 30.5.7 which recognizes a determinant in the N/C₁ domain of the $H-2L^d$ molecule¹². The immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Both the deleted and wild-type proteins are associated with β_2 -microglobulin. As expected, the molecular weight of the truncated $H-2L^d$ antigen is significantly smaller than the complete $H-2L^d$ gene product.

S₁ analysis of truncated H-2 transcripts

The fact that the protein is shorter does not guarantee that the mRNA has spliced correctly and that the protein has the expected sequence. To determine if the truncated gene product is the same as the predicted structure shown in Fig. 2, S₁ nuclease mapping was used to analyse total RNA obtained from transformants expressing the truncated gene product. This analysis was done using a single-stranded uniformly labelled DNA probe of high specific activity that was prepared by primer extension of a recombinant phage M13 DNA template. A BamHI-RI fragment derived from the murine $I-A\beta$ gene was cloned into the M13 phage (Fig. 4). After hybridizing mRNA with the labelled DNA probe, the reaction mixture was digested with S₁ nuclease and the products were analysed on denaturing polyacrylamide gels (Fig. 4). Only probes that had been hybridized to either mRNA from the transformed L-cells or from mRNA made

from B cells expressing the $I-A\beta$ gene were protected from S₁ nuclease digestion. An 89-nucleotide fragment was protected by mRNA isolated from clone D3.8 (Fig. 4, lane D). The protected fragment is identical to the fragment protected by mRNA (poly (A)⁺) isolated from A20, a B-cell lymphoma expressing the $I-A\beta$ gene (Fig. 4, lane D). Furthermore, the protected fragment is about the size (85 base pairs) predicted by comparison of the $I-A\beta$ cDNA sequence²¹ to the genomic $I-A\beta$ sequence¹⁶.

These results demonstrate that splicing of the cytoplasmic domain in the truncated $H-2L^d$ mRNA occurs similarly to that of the $I-A\beta$ mRNA normally expressed in a B-cell lymphoma, A20. Thus, the amino acid sequence of the COOH-terminus of the truncated $H-2L^d$ product is predicted in Fig. 2. The deleted gene product has removed 25 amino acid residues from the native $H-2L^d$ gene product and replaced these with three new residues.

Cytoplasmic part of H-2 in recognition

To determine the effects of deleting the cytoplasmic portion of the $H-2L^d$ gene, L-cells expressing the native and truncated $H-2L^d$ genes were used as targets for effector T cells in four systems. We and others have previously described the use of L-cells transformed with $H-2L^d$ genes as targets for killer T cells^{9–12}. Allo-specific T cells recognize L-cells expressing the truncated gene product nearly as well as they recognize the

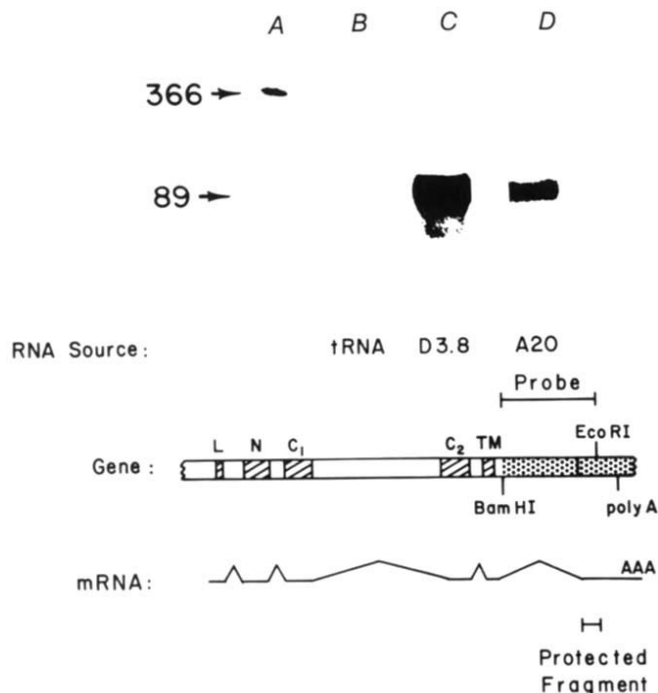


Fig. 4 Results of S_1 nuclease mapping analysis of mRNA isolated from clone D3.8 and B-cell lymphoma A20. **A** *Bam*HI-*Eco*RI fragment encoding the second cytoplasmic domain and 3' untranslated region of the murine *I-A β* gene was cloned into M13mp9. After transfection of *E. coli* JM103³⁸ appropriate recombinants, each containing the strand of the *I-A β* DNA that would serve as a template for the synthesis of a probe complementary to mRNA, were selected. Single-stranded DNA was recovered by using standard techniques after overnight incubation at 37°C³⁸. **A**, The probe fragment; **B**, tRNA hybridized with the probe and S_1 digested; **C**, mRNA from clone D3.8 hybridized and S_1 digested; **D**, mRNA from B-cell lymphoma A20 hybridized and S_1 digested. **Methods:** A universal 24 base long primer was annealed with 0.3 μ g of the single-stranded M13 recombinant; synthesis of the radiolabelled strand was then directed by the large fragment of DNA polymerase I using [α -³²P]dATP (400 Ci mmol⁻¹) and unlabelled dTTP, dCTP and dGTP. Synthesis of the second strand completed a unique *Hind*III site downstream from the insert. After synthesis of the second strand, digestion with *Hind*III released a ³²P-labelled single-stranded copy of the insert. This radiolabelled fragment was very different in size from the linear unlabelled template. The single-stranded radiolabelled probe was purified on a denaturing gel and recovered by electroelution³⁹. mRNA was isolated from cells as described previously⁴⁰. Samples containing 10 μ g total mRNA from clone D3.8, 10 μ g poly (A)⁺ mRNA from A20 and 20 μ g tRNA were hybridized overnight with 50,000 c.p.m. of single-stranded probe at 55°C in 30 μ l reaction mixture containing 75% (v/v) formamide⁴¹. Each reaction mixture was then digested with 700 U S_1 nuclease at 42°C for 30 min^{41,42} and the products were precipitated and analysed by electrophoresis on denaturing polyacrylamide sequence gels⁴³. The size of the protected fragment was measured at 89 bases by co-migration with ³²P-labelled *Hinf* digested pBR322.

L-cells expressing the normal gene product (Table 2). Furthermore, influenza virus specific CTLs recognized virus-infected L-cells expressing the deleted *H-2L^d* gene product nearly as well as L-cells expressing the native *H-2L^d* gene product. By contrast, L-cells expressing the deleted *H-2L^d* product and infected with VSV were recognized less efficiently by the VSV specific killer T cells as the L-cells expressing the native *H-2L^d* molecule and infected by the virus (Table 2).

L-cells expressing the deleted *H-2L^d* gene product (clone D3.8, Table 1) appeared to bind the same amount of monoclonal *H-2L^d* antibody as the T1.1.1 cell (*H-2L^d*). Nevertheless, the possibility remained that small differences in the amount of *H-2L^d* on the cell surface had a significant effect on the recognition of these cells by VSV specific CTLs, even though these

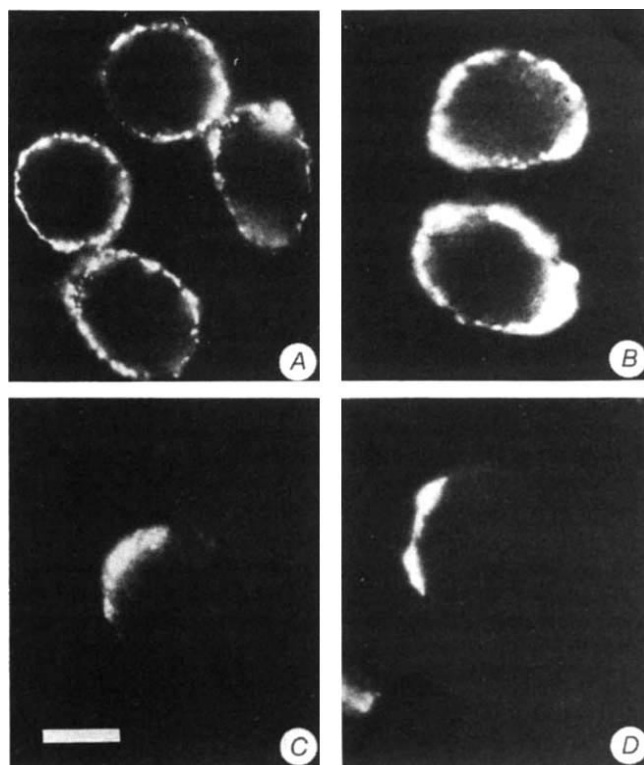


Fig. 5 T1.1.1 and D3.8 cells were detached from culture dishes with a rubber policeman and dissociated into individual cells with a Pasteur pipette in the cold room (4°C). Cells were incubated with diluted mouse monoclonal antibodies (mixture of 30.5.7 and 28.14.8) in a sealed tube containing RPMI 1640 medium at 4°C for 30 min. After washing in RPMI 1640 medium, cells were incubated with diluted fluorescein-conjugated goat anti-mouse immunoglobulin (Cappel) in RPMI 1640 medium at 4°C for 30 min. After washing, an aliquot of cells was placed under a glass coverslip, sealed with nail polish and examined immediately by Zeiss Photomicroscopy III equipped with epifluorescent optics (Planapo, $\times 63$). The rest of the cells were divided into two tubes, one kept at 4°C, the other at 37°C. At various times an aliquot of cells was removed and examined as above. For each time point, 300 cells were scored for redistribution of antigen. Photographs were made with Kodak Tri-X film (E1600) and developed in HC-110 dilution B for 12 min. Cells kept at 4°C did not have a significant antigen redistribution at the end of the experiments. **A**, T1.1.1 and **B**, D3.8 immediately after staining at 4°C. **C**, T1.1.1 and **D**, D3.8 after 2 h at 37°C. Scale bar, 20 μ m.

differences could not be measured by radioimmunoassay. To test this possibility we measured the ability of VSV specific CTLs to kill L-cells expressing significantly less native *H-2L^d* (Table 1, clone T123.8) than T1.1.1 cells (Table 1). These cells, expressing lower levels of *H-2L^d*, are killed just as effectively as cells expressing high levels of *H-2L^d* (Table 2). Thus, we conclude that the VSV specific CTLs are not able to recognize the truncated *H-2* molecules as efficiently as they can recognize VSV associated with the normal *H-2L^d* gene product.

Cytoplasmic part of *H-2L^d* in capping

The *H-2* molecule is normally uniformly distributed across the cell surface. Shortly after the addition of a monoclonal *H-2* specific antibody and a fluorescent-labelled second cross-linking antibody, the *H-2* molecules on the cell surface start to redistribute. Initially this redistribution involves the aggregation of the *H-2* molecules into patches, presumably through the cross-linking of *H-2* molecules by antibodies in the plane of the membrane. This event is followed by movement of these patches into a polar cap^{22,23}. Other investigators have proposed that the latter is an energy-dependent process resulting from

association of transmembrane glycoproteins with cytoskeletal elements^{24,25}. We investigated the nature of the interaction between the H-2 molecule and cytoplasmic proteins by studying the ability of the truncated H-2L^d molecule to make caps on the cell surface. Figure 5 shows the 'patching' and 'capping' of H-2L^d molecules expressed on the surface of T1.1.1 (complete H-2L^d genes) and D3.8 cells (truncated H-2L^d gene) after reaction with monoclonal antibody and fluorescent-labelled second antibody. Initially, the H-2L^d molecules are evenly distributed over the entire surface of the plasma membrane (Fig. 5A, B). After 2 h incubation the patches coalesce into a single cap on the surface of the T1.1.1 (Fig. 5C) and D3.8 (Fig. 5D) cells. To investigate if the rate of capping of the truncated H-2L^d antigen was different from the native molecule, a time course experiment was performed (see legend to Fig. 5). After 30 min incubation at 37 °C, 25% of T1.1.1 and 20% of D3.8 cells had capped. After 1 h at 37 °C, 50% of T1.1.1 and 46% of D3.8 cells had formed caps. After 1.5 h, 70% of T1.1.1 and 74% of D3.8 had capped. After 2 h at 37 °C, 80% of the T1.1.1 and 85% of the D3.8 cells had capped. Apparently the deleted H-2L^d gene product caps at precisely the same rate as the native H-2L^d molecule. Thus, we suggest that the interactions of the intracytoplasmic portion of the H-2 molecule with other proteins do not have a significant role in the movement of cell-surface antigens into caps.

Conclusions

We have constructed an H-2L^d gene whose product lacks the intracytoplasmic portion. The shortened protein is expressed on the cell surface. The product of this truncated H-2 gene is efficiently recognized by allo- and influenza-specific cytotoxic T lymphocytes. Although VSV specific CTLs do not recognize this truncated H-2L^d molecule as efficiently as the complete H-2L^d product they are still able to recognize the truncated H-2 antigen. Thus, many of the cellular functions of the H-2 antigen can be performed by the truncated molecule. These findings suggest that interactions between the cytoplasmic proteins of the cell and the H-2 molecule do not have a critical role in the function of the H-2 molecule.

The nature of the interaction between viral antigens and H-2 antigens on the cell surface has puzzled immunologists for a long time. Several studies have indicated that some viral antigens and H-2 antigens are co-precipitated²⁶⁻²⁸ or co-capped^{29,30} by

the appropriate antiserum. By contrast, no association has been identified between influenza virus antigens and H-2 antigens³¹. Presumably, these different results reflect differences in the interactions between particular viral antigens and the H-2 antigens. These differences might also account for the differences in the ability of the VSV specific CTLs and influenza specific CTLs to recognize cells expressing the truncated H-2 antigen. If interactions between viral and H-2 antigens do occur, these interactions must be quite subtle. Thus, small differences in experimental conditions may have a significant effect on the apparent function of an H-2 molecule. The weakness of these interactions also raises the question of which of these interactions are important to the animal rather than those detected in tissue-culture conditions. The introduction of the truncated H-2 gene described here directly into the mouse genome may allow a direct test of this question.

The truncated H-2L^d gene product and the wild-type H-2L^d molecule function identically in most of the cellular assays, suggesting that the structure of the protein is not grossly altered by the deletion of the cytoplasmic portion. To test whether a physical parameter, the ability of these molecules to form caps, is affected by changes in the cytoplasmic portion we compared the rates of the cap formation on cells expressing the two proteins. We observed no differences in the rate of capping between the truncated and complete H-2L^d antigen. We conclude that if interactions between other cellular proteins and the intracytoplasmic portion of the H-2 molecule have an important role in cap formation, then these interactions are non-specific. Co-capping experiments of viral antigens and truncated H-2L^d molecules will be examined to determine if the truncated H-2L^d is able to co-cap viral antigens as well as the wild-type antigen co-caps viral antigens.

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Note added in proof: Zuniga *et al.*⁴⁴ recently reported the synthesis and characterization of an H-2 gene encoding an H-2 molecule similar to the one described here.

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Tripartite structure of the *Brassica campestris* mitochondrial genome

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Physical mapping studies indicate a novel sequence arrangement, consisting of three circular chromosomes, for the mitochondrial genome of Brassica campestris (Chinese cabbage, turnip). A master chromosome, 218 kilobases (kb) long, contains the entire sequence complexity of the mitochondrial genome, including two copies of an ~2-kb element present as direct repeats separated by 135 and 83 kb. The two smaller circles are 135 and 83 kb in size, contain one copy each of the 2-kb repeat element, and are postulated to interconvert with the master chromosome via a co-integration-resolution pathway mediated by reciprocal recombination within the 2-kb repeat.

HIGHER plant mitochondrial genomes are unusually large and variable in size, ranging from ~200 kilobases (kb) in *Oenothera*¹ and *Brassica*^{2,3} to almost 2,500 kb in muskmelon⁴. The way in which this DNA is organized into discrete molecules is unclear⁵⁻⁷. Electron microscopic analysis of mitochondrial DNA isolated from native plant tissue reveals a broad size range of molecules, primarily linear with a low percentage of circles⁸⁻¹⁰. A much larger percentage of the mitochondrial DNA from cultured cells can be isolated as intact circles, with predominant size classes in the range 2–30 kb¹¹⁻¹³. Several models have been proposed which attempt to reconcile the large kinetic and restriction complexities of plant mitochondrial DNA with the failure to find a discrete size class of molecules large enough to accommodate this information (see refs 5 and 6 for a review). At one extreme, it has been proposed that the plant mitochondrial genome may exist as a collection of different size classes of circular molecules, with each size class containing a distinct, non-overlapping subset of the total sequence complexity of the genome¹⁴. Alternatively, it has been suggested that the genome is organized primarily as a single large linkage group, with frequent recombination and rearrangement leading to the array of smaller molecules more easily isolated and observed via electron microscopy¹⁵. Data in support of these and other models are hardly overwhelming, although the last model is consistent with restriction mapping of corn mitochondrial DNA¹⁶⁻¹⁸, where several linear linkage groups have been determined that are larger than any of the observed circular molecules⁹.

We now report the first complete restriction map for a higher plant mitochondrial genome, that of *Brassica campestris* (Chinese cabbage, turnip). The model most consistent with our mapping data is that this genome is organized into three physically distinct circular molecules. The largest circle of 218 kb bears the entire sequence complexity of the genome and thus constitutes a 'master' chromosome, and the two smaller circles contain distinct, 135-kb and 83-kb subsets of the master molecule. These three chromosomes seem to interconvert via reciprocal recombination across the major repeat element present on all three molecules.

Physical mapping studies

A clone bank containing 96% of the *B. campestris* mitochondrial genome was constructed by inserting *Pst*I and *Sal*I fragments into the plasmid vector pUC8 (ref. 19). Each of these cloned fragments was labelled with ³²P and hybridized to nitrocellulose filters containing single digests of total mitochondrial DNA with each of five enzymes (*Pst*I, *Sal*I, *Bgl*I, *Kpn*I, *Nru*I) and also selected double digests with various combinations of these en-

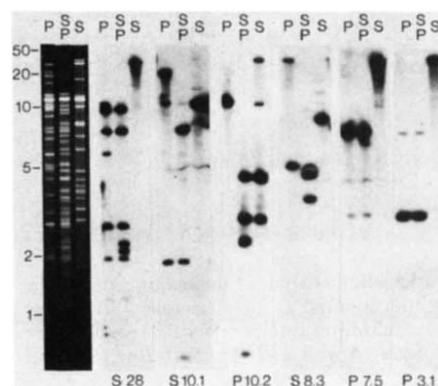


Fig. 1 Representative mitochondrial DNA mapping hybridizations. Left-hand panel: DNase I-purified²⁵ mitochondrial DNA from *B. campestris* was digested with *Pst*I (P), *Sal*I-*Pst*I (SP) and *Sal*I (S), and fragments were separated by electrophoresis in a 0.7% agarose gel³⁸. The size scale on the left is in kb and was determined using λ phage DNA restriction fragment markers generated with *Sal*I, *Hind*III and *Eco*RI. Right-hand panels: six cloned mitochondrial DNA restriction fragments were each labelled with ³²P by nick-translation³⁹ and hybridized³⁸ to a replica nitrocellulose filter imprint³⁸ of the gel shown in the left panel. In this and the other figures, and also in the text, a given mitochondrial DNA restriction fragment, or a plasmid containing the fragment, is designated both by a letter, indicating the enzyme used to generate the fragment (P = *Pst*I, S = *Sal*I, B = *Bgl*I, K = *Kpn*I, N = *Nru*I) and a number, indicating the size of the fragment in kb.

zymes. The mapping strategy is illustrated in Fig. 1. A cloned *Sal*I fragment of 28 kb (S28) hybridized to eight *Pst*I fragments, ranging in size from 10.2 to 0.9 kb (Fig. 1). Hybridization to a *Sal*I-*Pst*I double-digest lane indicated that six of these fragments (9.7, 7.5, 2.9, 2.0, 1.3 and 0.9 kb in size) are uncut by *Sal*I, and thus lie completely internal to S28, while P10.2 and P5.7 are cut by *Sal*I and thus overlap the junctions between S28 and two flanking *Sal*I fragments (Fig. 1). P10.2 hybridized to a double-digest fragment of the same size (2.4 kb) as one of the two terminal S28 double-digest fragments. We conclude, therefore, that this fragment represents the overlap between S28 and P10.2, and that the other terminal S28 double-digest fragment, of 2.2 kb, must represent its overlap with P5.7. P10.2 also hybridized to internal *Sal*I fragments of 4.4 and 2.8 kb and to a second *Sal*I fragment, S10.1, cut by *Pst*I (Fig. 1). The length of the overlap between P10.2 and S10.1 is given by the 0.6-kb double-digest fragment to which they both hybridize (Fig. 1). Thus, using only three hybridizations, a region of 60 kb of DNA, from P19.4 to P5.7, was mapped for these two enzymes (Fig. 2).

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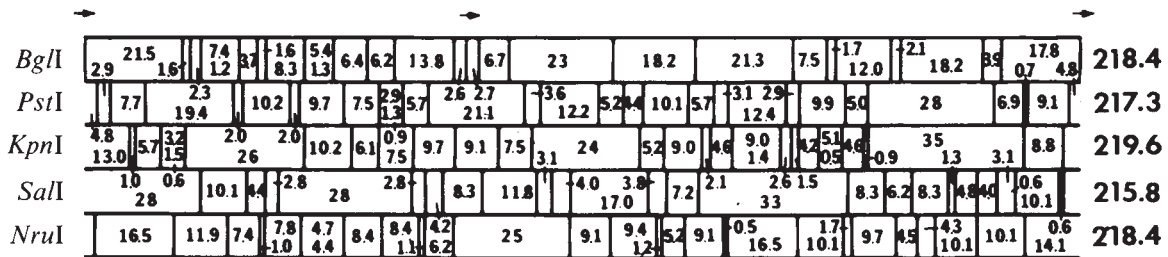


Fig. 2 Restriction map of the master chromosome of *B. campestris* mitochondrial DNA. The circular map for the largest mitochondrial linkage group, containing the entire sequence complexity of the genome, is shown linearized at the *BglI* site internal to the 2-kb repeat sequence. Arrows indicate the position and relative orientation of the two copies of the 2-kb repeat present on this linkage group. The numbers at the right-hand end of the map indicate the summation of the restriction fragments for each of the enzymes marked at the left-hand end.

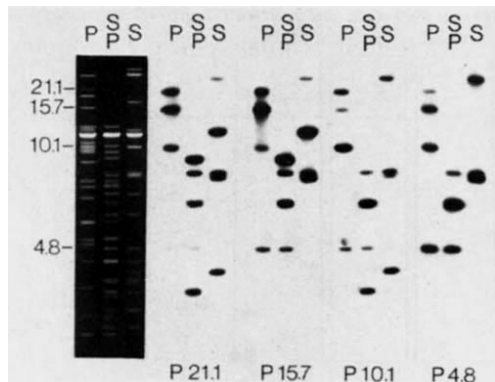


Fig. 3 Hybridization analysis of the major repeat sequence of *B. campestris* mitochondrial DNA. The four *PstI* clones indicated were each hybridized to replica nitrocellulose blots of mitochondrial DNA digested with *PstI* (P), *SalI*-*PstI* (SP) and *SalI* (S).

In control *PstI*-*PstI* or *SalI*-*SalI* hybridizations, several cloned fragments hybridized to one or more fragments in addition to the expected fragment of the same size of the cloned insert. Except for one special case, described in detail below, these extra hybridizations were all very weak and did not confuse interpretation of the hybridization results in terms of constructing a map. In several cases, reciprocal cross-hybridizations clearly indicated that these weak hybridization signals result from small repeated sequences present in two or more copies in the mitochondrial genome (compare P7.5 and P3.1 hybridizations in Fig. 1). In other cases, the weak, additional hybridizations were to fragments larger than the probe fragment and sometimes could not easily be assigned to major fragments visible in the stained gel (see S10.1 and S8.3 hybridizations in Fig. 1). These hybridization signals could result either from slight partial digestion products, from the presence of minor repeat elements within the major bands, or from microheterogeneity in the DNA, resulting in very faint bands not clearly visible in the stained gel.

2-kb Repeat element

The major cross-hybridization between cloned fragments was observed with four *PstI* fragments, each of which hybridized to itself and to the other three *PstI* fragments (Fig. 3). The sequence common to these four fragments has a minimum size of 0.6 kb, as defined by common *Bam*HI and *Bgl*I sites, and a maximum possible size of 3.2 kb, as defined by *Eco*RI digestion (Fig. 4). Hybridization of each of the four *PstI* fragments to *Bam*HI and *Bgl*I digests of all four clones (normalizing insert cross-reactions to vector hybridizations) allows a very rough quantitative estimate of the size of this repeat as 2 ± 1 kb. For convenience, this element will henceforth be referred to as the '2-kb repeat'.

Three of the 2-kb repeat-containing *PstI* fragments, P21.1, P15.7 and P4.8, are clearly substoichiometric relative to the rest of the *PstI* fragments as judged both by direct visualization of stained gels (Figs 1, 3) and densitometric scanning of a *PstI*

digest (Fig. 5). The fourth 2-kb repeat-containing fragment, P10.1, co-migrates with a second, non-identical fragment and its stoichiometry could not be evaluated directly. It should be emphasized that, taking into account degradation effects on the larger fragments, all the remaining *PstI* fragments fall into a unimolar stoichiometric series except for one doublet (P5.7), one triplet (P2.9), and a bright band at 11.3 kb which corresponds to an extrachromosomal, linear mitochondrial DNA plasmid described previously³.

The gels and gel scan suggest that P21.1 and P4.8 are present in submolar amounts relative to P15.7 (Figs 1, 3, 5). Direct support for this conclusion can also be gained from quantitative consideration of the hybridizations shown in Fig. 3. If P21.1 and P15.7 were present in equal molarity within the mitochondrial genome, then, assuming that single-stranded probe lengths are short relative to insert sizes, a P21.1 probe should hybridize to itself approximately 1.5 times (21.1 divided by 14—the approximate size of the region of homology between P21.1 and P15.7; see Fig. 4) more intensely than to P15.7, while a P15.7 probe should hybridize to itself only 1.1 times (15.7/14) more intensely than to P21.1. In fact, visual inspection reveals that P21.1 hybridizes to itself somewhat less intensely than to P15.7, while P15.7 hybridizes to itself significantly more intensely than to P21.1 (Fig. 3). Quantitation of these hybridization signals by densitometry and measurement of the area under each curve indicates that P15.7 is actually present in about twice the molarity of P21.1. These calculations can only be approximate for two reasons. First, the size of the repeat is not accurately known, and second, significant degradation of the DNA on the filter results in diminished hybridization signals for the large fragments relative to small ones. Similar pairwise comparisons of the hybridizations in Fig. 3 lead us to conclude that P10.1 is also present in approximately twice the concentration of P21.1, and that P15.7 and P10.1 are approximately equimolar and are approximately twice as abundant as P4.8. Thus, we conclude that the four *PstI* fragments which contain the 2-kb repeat are all submolar and that they can be further subdivided into two groups, with P15.7 and P10.1 being present in approximately equal molarity and at roughly twice the concentrations of P21.1 and P4.8.

Multicircular mitochondrial DNA

The 2-kb repeat is present on all four substoichiometric *PstI* fragments. In addition, each *PstI* fragment is identical, on one side of the repeat, to one of the other three fragments and on the other side of the repeat to a second one of the other three (Fig. 4). In other words, the 2-kb repeat is flanked by four paired combinations of four unique sequences. The two lower molarity substoichiometric fragments, P4.8 and P21.1, are equivalent in sequence complexity to the higher molarity pair, P15.7 and P10.1, and the two pairs are interconvertible by a single reciprocal cross-over within the 2-kb repeat.

The overall arrangement in the genome of these four substoichiometric, repeat-containing *PstI* fragments was determined by completing the chromosomal 'walk' described above. These hybridizations generate a single, circular linkage group

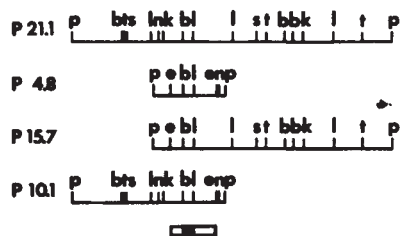


Fig. 4 Restriction maps of the four, 2-kb repeat-containing, substoichiometric *PstI* fragments of *B. campestris* mitochondrial DNA. Cleavage sites for *Bam*HI (b), *Bgl*II (l), *Bst*EII (t), *Eco*RI (e), *Kpn*I (k), *Nru*I (n), *Pst*I (p) and *Sal*I (s) were mapped by appropriate double and triple digestions of plasmids containing each of the four *Pst*I fragments indicated. All cleavage sites are shown for all enzymes except for *Eco*RI, which produced many small fragments with the three largest clones. However, as *Eco*RI cleaves P4.8 only twice, defining the maximum boundaries of the repeat sequence as presently mapped, we show these two sites as they occur on P4.8, P10.1 and P15.7. The repeat sequence (bl) is flanked by four paired combinations of four unique sequences (pbtslnk, lsbtkltp, pe, enp). The solid bar indicates the minimum extent of the repeat sequence and open extensions of the bar its maximum extent.

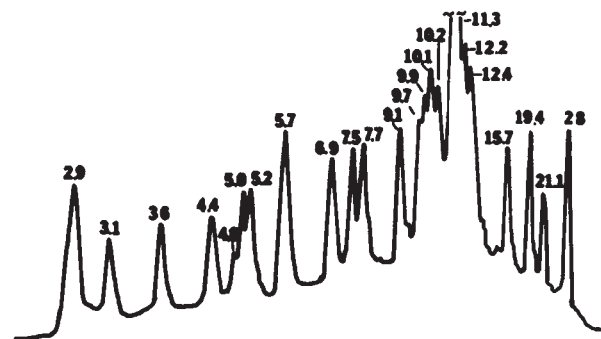


Fig. 5 Stoichiometry of *Pst*I fragments of *B. campestris* mitochondrial DNA. A negative of a gel similar to those shown in Figs 1 and 3 was densitometrically scanned in a Beckman DU spectrophotometer at an absorbance wavelength of 600 nm.

(Fig. 2) that accounts for every fragment for all five enzymes except for two fragments with each of *Pst*I (P15.7 and P10.1), *Kpn*I (K11.0, a doublet), *Nru*I (N35 and N3.9) and *Sal*I (S28 and S8.1). This circle contains the entire 218-kb sequence complexity of the *B. campestris* mitochondrial genome, including two copies of the 2-kb repeat present in direct orientation within P21.1 and P4.8 (Fig. 2). When the chromosomal walk is started at P15.7 or P10.1 and continued outwards in both directions, smaller circular linkage groups are generated, of 135 and 83 kb, respectively, each containing just one copy of the 2-kb repeat (Fig. 6).

The *Kpn*I, *Nru*I and *Sal*I fragments not accounted for by the large circle (Fig. 2) are those that span the 2-kb repeat on the two small circles (Fig. 6). It was not possible to evaluate the stoichiometry of the repeat-containing *Sal*I fragments (Figs 1 and 3) and *Kpn*I fragments due to their co-migration with other fragments. However, all four of the repeat-containing *Nru*I fragments are well separated and appear substoichiometric with approximately the same molarity relationships as described for the corresponding *Pst*I fragments (data not shown). *Bgl*II cuts within the repeat (Figs 2, 4, 6), thus no substoichiometric *Bgl*II fragments were observed.

Together, the two small circles contain the entire sequence complexity of the master chromosome. Figure 6 presents a simple model for the interconversion of these three circles via reciprocal recombination between directly oriented 2-kb

repeat elements. Two recombination reactions are shown: intramolecular recombination between repeat elements present on the master chromosome will lead to its resolution into the two small circles, and the converse, intermolecular recombination between 2-kb repeats on the small circles will lead to their cointegration to form the master circle. This model portrays only two of essentially an infinite number of possible recombination reactions. Each of the two small circles could recombine with either repeat element on the large circle, generating circles of 353 or 301 kb. Recombination could occur between identical circles, leading to circular dimers. Higher order reactions could involve any of these multimeric molecules and also the simpler molecules shown in Fig. 6, leading to larger and larger circles, *ad infinitum*.

The simplest alternative to the three-circle model shown in Fig. 6 is a circular dimer model^{20,21}. Head-to-head 436-kb circular dimers formed by intermolecular recombination between the P4.8 repeat present on one 218-kb circle and the 21.1-kb repeat present on a second circle would contain all four of the repeat orientations shown in Fig. 6. However, a collection of 218-kb monomers and head-to-head dimers would have higher levels of the P4.8 and P21.1 repeats (present on both monomers and dimers) than of the P15.7 and P10.1 repeats (present on dimers only). Even in the limit case, where all of the mitochondrial DNA existed as head-to-head 436-kb dimers, the four repeats would be equimolar. As P15.7 and P10.1 are approximately twice as abundant as P4.8 and P21.1, we conclude that the simplest model consistent with both mapping and stoichiometry relationships is the three-circle model (Fig. 6). While we believe it likely that most of the mitochondrial DNA exists in one of these three circular forms, this does not preclude a small percentage of the DNA existing as circular dimers or

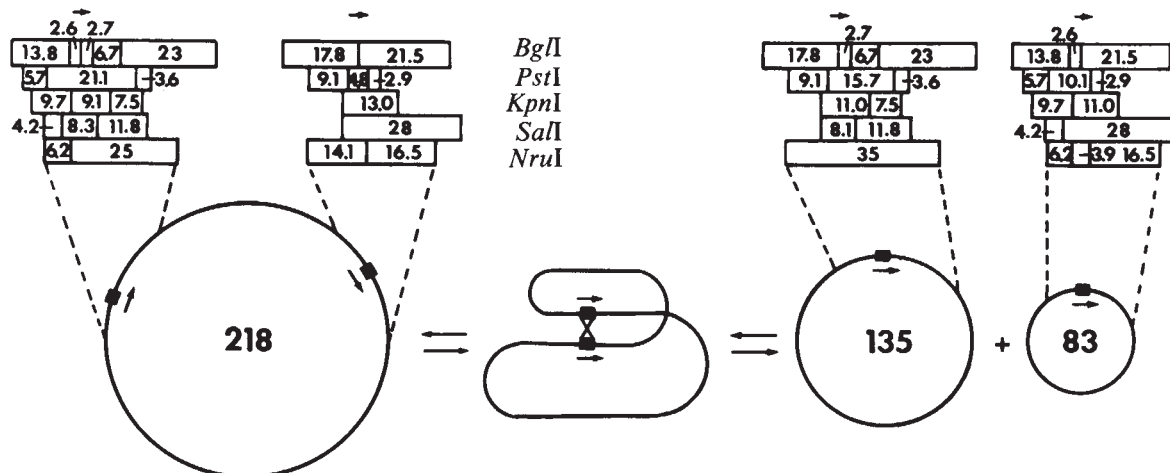


Fig. 6 Top, restriction maps of the four regions of the mitochondrial genome which contain the 2-kb repeat sequence. Bottom, predicted organization of the *B. campestris* mitochondrial genome into three circular molecules and their interconversion via recombination within the 2-kb repeat. Solid boxes indicate the position of the repeat and arrows its orientation.

any of the other multimers described in the preceding paragraph.

Regardless of the ultimate size of the molecules on which they are present, P4.8 and P21.1 seem to be inextricably physically linked and thus must be present in equal molarity. As P15.7 and P10.1 are present at roughly twice the concentration of P21.1 and P4.8, it can be concluded that there are approximately two molecules of the two small circles for each molecule of the master chromosome. That P15.7 and P10.1, and thus the circles they represent, are approximately equimolar suggests that the master chromosome may be the replicating molecule within the mitochondrion. According to this hypothesis, most recombination events would occur intramolecularly, generating the two small circles from the master chromosome, and thereby maintaining equal levels of these two circles. Clearly, however, fragment stoichiometries, approximate as they are, can provide only suggestive evidence concerning the direction of recombination and replication, and more direct experimental tests are necessary to provide conclusive answers.

Discussion

The organization of the *B. campestris* mitochondrial genome into three physically distinct circular chromosomes maintained via recombination across a common repeat element is a novel pattern of mitochondrial DNA sequence organization. While a great diversity of small extrachromosomal molecules have been found in the mitochondria of plants and fungi, the main mitochondrial genome in all eukaryotic organisms characterized to date consists of a single molecular species, usually circular, although occasionally linear^{6,7,22}.

Is the tripartite structure of mitochondrial DNA from *B. campestris* typical of other plant mitochondrial genomes, most of which are considerably larger? Sets of four repeat-containing restriction fragments having paired combinations of unique flanking sequences, completely analogous to those characterized here, have been found in corn²¹, wheat²³ and spinach (D. B. Stern and J.D.P., unpublished data). In corn, extensive restriction mapping of the 569-kb mitochondrial genome indicates that the bulk of the genome may be organized in a tripartite fashion similar to that of *Brassica*, although secondary recombination events outside the major repeat sequence appear to generate an extra diversity of circular size forms not seen in *Brassica* (ref. 21 and D. M. Lonsdale, personal communication). Recent work also indicates that similar processes of intramolecular recombination and excision may lead to the dissolution of the 65-kb *Neurospora* mitochondrial genome into 22- and 43-kb circles in certain *stopper* mutants²⁴.

The process of recombination between repeated sequences to give alternate states of *Brassica* mitochondrial genome organization—either two small circles or one large co-integrate circle—is analogous to the process of λ phage integration—excision into the *Escherichia coli* chromosome²⁶ and that of bacterial transposable element cointegrate formation—resolution²⁷. By analogy to these and mechanistically related specialized site-specific recombination systems^{28–31}, we speculate that

there may be a specialized recombination system, possibly encoded within the *Brassica* mitochondrial genome and perhaps even physically linked to the 2-kb repeat sequence, which mediates recombination between specific recombination sites embedded within the 2-kb repeat. We are now examining these hypotheses through sequence studies of the 2-kb repeat and by testing its ability to act as a substrate in a heterologous site-specific recombination system³².

Further analogy, to processes of low-frequency, secondary site recombination between λ DNA and the *E. coli* chromosome²⁶, leads to the prediction that recombination may also occur between the putative strong recombination site located in the 2-kb *Brassica* repeat and weaker recombination sites located elsewhere in the genome. This possibility will be studied in *Brassica*, and may be related to the high levels of small circular mitochondrial DNA molecules observed in suspension cell cultures of other plant species^{11–13}. We hypothesize that these abundant small circles may result from secondary recombination processes which normally occur at very low frequency in native plant tissues but which are intensified when plant cells are cultured *in vitro*.

The *B. campestris* mitochondrial genome is relatively small compared with the mitochondrial genomes of most other higher plants. Therefore, we expect that it will serve as an excellent model system in which to examine many fundamental questions concerning the structure, expression and function of plant mitochondrial genes and also the precise involvement of the mitochondrial genome in cytoplasmic male sterility^{3,5,6}. In addition, important evolutionary questions relating to the mode and tempo of plant mitochondrial DNA divergence can now be directly addressed. It is well known, for example, that animal mitochondrial DNA evolves extremely rapidly at the primary sequence level but is highly conserved in gene arrangement, whereas fungal mitochondrial DNA is characterized by a high degree of genomic rearrangement^{7,33}. In contrast, little is known about how plant mitochondrial DNA evolves, although limited observations suggest that it may be relatively conserved among mitochondrial DNAs at the sequence level, with a moderate tendency to rearrange^{7,34}. We are now addressing these questions by comparing restriction maps of mitochondrial DNA from *B. campestris* and from five other species in the genus *Brassica*. Evolutionary divergence values are well established for the chloroplast DNAs of these species³⁵, thus these experiments should also give valuable information on relative rates of sequence change in the two cytoplasmic organelles. Finally, we point out the utility of a complete physical map for furthering our understanding of the nature and pattern of integration of chloroplast DNA sequences within plant mitochondrial DNAs^{17,36,37}.

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On the far-infrared excess of Vega

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The Infrared Astronomy Satellite (IRAS) has recently detected a compact far-infrared emission source associated with Vega (α Lyrae)¹. This flux appears to be thermal emission from dust heated by the visual/UV luminosity of Vega. Although circumstellar dust is commonly associated with young, early-type stars², the characteristics of the emission from Vega are quite atypical. The essential difference is the relatively low temperature of the dust for the observed source size. This low temperature implies that the dust grains producing the far-infrared emission must be very efficient 60/100- μ m emitters and must, therefore, be much larger than typical interstellar and circumstellar dust grains. To place more limits on the properties of this far-infrared emission region, we observed Vega at 47 and 95 μ m with the Kuiper Airborne Observatory (KAO) on the night of 12–13 August 1983. We report here confirmation of the IRAS results and that the source size may be as large as 46 arc s along some axis.

We used a 3 spatial $\times$ 2 spectral element array radiometer described in detail by Wilking *et al.*³. Briefly, this system measures simultaneously the flux at two wavelengths from three circular regions of sky which lie along a line in cross-elevation. The centre-to-centre separation between the beams is 1 arc min. The cross-elevation direction was rotated 15° to the north-west and south-east from a line parallel to declination. We observed at 47 μ m ($\Delta\lambda = 20$ μ m) and at 95 μ m ($\Delta\lambda = 80$ μ m). In addition to the normal short-wavelength blocking provided by a diamond scatter filter and various crystalline materials^{4,5}, we added a layer of 0.003-inch black polyethylene on the outside of the radiometer to eliminate any possible short-wavelength leaks in the standard filtering. The beams are roughly gaussian in shape with half-power widths of 30 and 43 arc s respectively at 47 and 95 μ m. The oscillating secondary chopper of the KAO was operated with a throw of 3 arc min in cross-elevation.

The central of the three beams was placed on the visible image of Vega so that the outer beams observed points roughly 1 arc min to the north and south. The measured flux densities in all six channels after about 4,000 s integration time are listed in Table 1. The observations were calibrated relative to IRC+10420. We have measured the flux density of IRC+10420 relative to Mars, S140 (ref. 6), and NGC7027 (ref. 7), on several flights and find values of $1,270 \pm 135$ and 360 ± 70 Jy, respectively at 47 and 95 μ m. (These measurements have also refined our estimates for the flux density of S140 to 6,600 and 6,900 Jy respectively.)

It is clear from Table 1 that far-infrared flux is definitely seen from Vega at a level similar to that reported by Aumann¹. It is also apparent that no flux was seen in the detectors which viewed positions 1 arc min away from the star to a 2σ limit of roughly one-third that seen on Vega itself. A more stringent limit on the far-infrared surface brightness at a radius of 1 arc min from Vega may be obtained by averaging the four measurements taken off the peak. This leads to an average 50–100 μ m

flux density of 0.45 ± 0.35 Jy, a factor of 10 or more fainter than the central value. Therefore, the source radius is certainly less than 1 arc min, consistent with the results of Aumann *et al.*¹.

If we combine our subarc min resolution data with those of Aumann *et al.*¹, we can place better limits on the source size. For comparison, we have calculated the flux densities at our effective wavelengths of 47 and 95 μ m using the IRAS flux densities and assuming a black-body spectral shape. These are then 8.2 Jy at 47 μ m and 7.3 Jy at 95 μ m. Our observed flux densities (Table 1) are 0.56 ± 0.15 and 0.62 ± 0.21 of the IRAS values at 47 and 95 μ m, where the errors include all our uncertainties and a nominal 10% error for the IRAS data. Within 3σ error limits these are consistent with unity. But we may derive a rough estimate of source size by assuming that the lower flux densities seen by our smaller beams result because the source size is comparable to our beam size. In this case, the diameter of a uniform disk large enough to produce the above flux ratios is 42^{+10}_{-7} arc s and 53^{+18}_{-10} arc s, respectively, at 47 and 95 μ m. Although the formal error limits on the source size are quite large, the fact that our observed colour temperature of 79 K is less than the IRAS value of 85 K, is also consistent with the idea that the source size is comparable to our beam size. In such a case, the larger 95 μ m beam would be expected to measure a greater fraction of the total flux than the 47 μ m beam, leading to a lower colour temperature than found by IRAS. In the following discussion we will assume a source diameter of 46 ± 10 arc s based on our flux measurements.

This small source size implies dust grains which have an absorption efficiency at visual–UV wavelengths nearly equal to their emission efficiency in the far-infrared as noted already by Aumann *et al.*¹. If we equate the energy absorbed by grains at a radius of 23 ± 5 arc s with the energy re-emitted, we find that the ratio of Planck-averaged absorption efficiency to emission efficiency is 2.8 ± 1.3 . This is in extreme contrast to typical values for interstellar dust of 100–1,000 (refs 8, 9 and P. Makinen, N. J. Evans, P. M. H. and M. A. Kutner, in preparation). This near equality of visible and far-infrared efficiencies implies that the 47 μ m and 95 μ m emission efficiencies are almost certainly equal except for any small effects dependent on dust composition. Therefore, the IRAS colour temperature of 85 K is probably quite close to the physical dust temperature.

This high emission efficiency places strong lower limits on the size of the grains seen in emission. No calculations of $\lambda \sim 100$ μ m emission efficiency for 'large' grains have been found in the literature, but a rough estimate may be obtained from calculations of small grain properties at shorter wavelengths. For instance, the data of Aannestad¹⁰ show for typical interstellar grains that as soon as the wavelength becomes larger than 1–1.5 times the grain diameter, the emission efficiency drops rapidly from unity. Scaling to $\lambda \sim 100$ μ m, we expect the grains around Vega to have a diameter $d \geq 20$ μ m in order to have a far-infrared emission efficiency as large as one-third the short-wavelength absorption efficiency.

We may estimate the minimum mass necessary to produce the observed emission assuming the particles have unit emissivity, a radius $r \geq 10$ μ m, and density of 2 g cm⁻³. This mass is $M = 10^{24}$ g, comparable to the mass of a small satellite in the solar system. The mass required to produce the observed level of emission scales linearly with assumed particle size. Therefore, although it is possible to produce the observed flux density with a single large 'particle', the required mass is of the order of $10^6 M_{\odot}$.

The above limit on grain size, $d \geq 20$ μ m, is a very strong limit based only on the assumption that the emission is thermal and that the grain emissivity is a reasonable function of wavelength. An additional size limitation is imposed by the Poynting–Robertson effect¹¹ which will cause grains of diameter 20 μ m and density 2 g cm⁻³ to spiral in to the central star in a time of 8×10^6 yr, assuming $L_{\star} = 58 L_{\odot}$ (ref. 12), a source radius

Table 1 Observed flux densities (Jy)

	1 min South	Central	1 min North
47 μ m	0.55 ± 0.6	4.6 ± 0.85 (± 1.25)	0.45 ± 0.65
95 μ m	0.9 ± 0.65	4.5 ± 0.8 (± 1.40)	-1.1 ± 1.45

Absolute calibration uncertainty $\pm 20\%$ at 50 μ m; $\pm 25\%$ at 100 μ m. The total uncertainty, absolute plus statistical, is shown in parentheses.

$r \sim 180$ AU and a distance for Vega of 8 pc (ref. 13). Aumann *et al.*¹ have summarized a number of arguments which imply that the grains must have been produced at the time of formation of Vega. Therefore, the grains must be even larger than $d = 20 \mu\text{m}$ if the age of Vega is $>10^7$ yr.

We have confirmed the far-infrared excess of Vega found by the IRAS survey. From a comparison of flux densities in our beam relative to the IRAS data we find evidence that the source has a diameter of the order of 46 arc s. This is roughly twice that found by IRAS using deconvolution of larger beam data. Resolution of this disagreement must await further observations: it may result either from observational errors or from a source brightness distribution which is more complicated than the assumed uniform, spherical geometry.

These data also confirm the fact that the grains around Vega must be much larger than typical interstellar grains ($a \sim 0.1 \mu\text{m}$). Therefore, some form of grain growth must have occurred. The Poynting–Robertson effect places a limit on the grain radius a (μm) and the time since grain formation in units of 10^6 yr, t_6 , such that $a \sim t_6$ for a cloud diameter of 46 arc s. Further observations of the Vega dust cloud and theoretical modelling of grain growth should provide important insight into the kind of processes which may have led to the even greater ‘grain’ growth in the Solar System.

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$^{15}\text{N}/^{14}\text{N}$ ratio of dissolved N_2O in the eastern tropical Pacific Ocean

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Nitrous oxide (N_2O) is of prime interest for understanding stratospheric chemistry¹ and the energy budget of the troposphere². Atmospheric N_2O has been considered to be mainly regulated by biological processes in soils^{3–6}. However, the oceans have an important role in the geochemical cycle of N_2O both as a reservoir and a reaction site^{7–12}. We report here the first ^{15}N abundance data on dissolved N_2O collected from the eastern tropical Pacific Ocean where active nitrogen transformation has been proposed to occur^{12–16}. Our ^{15}N data provide additional evidence for the hypothesis that the subsurface waters act as a source of N_2O and that the extremely oxygen-depleted waters act as a sink.

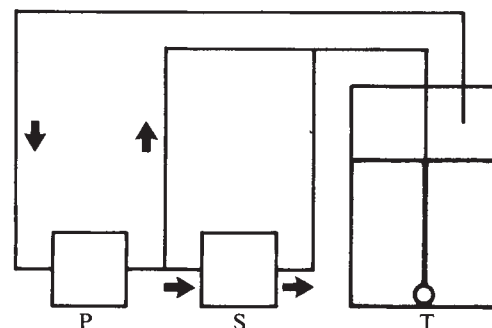


Fig. 1 The device used for extraction and collection of dissolved N_2O from seawater samples. Approximately 100 l of seawater was introduced into a stainless steel tank (T, ~ 150 l in volume), which had been filled with Ar. The gas above the sample water was circulated by the aid of a pump (P) through a sintered glass ball to facilitate emission of N_2O into the gas phase. A portion of the circulation gas goes through a bypass where N_2O is adsorbed on a molecular sieve 5A column in a sampling device (S) whose detail was described in ref. 17. Complete collection of N_2O was achieved within 12 h. For safety, the circulation was continued for 15 h.

Yoshida and Matsuo¹⁷ first introduced the nitrogen isotope ratio to assess the geochemical cycle of N_2O . In their study, they maintained that basic knowledge of the nitrogen isotope ratio of dissolved N_2O in the oceans would improve our understanding of the global budget of N_2O . The existing data on dissolved N_2O concentration in oceanic waters suggest that N_2O is produced in association with bacterial nitrification in subsurface waters and consumed by denitrification occurring in extremely oxygen-depleted waters^{8,9,11–13}. Low oxygen tension favours the production of N_2O associated with nitrification^{18–20}. We therefore focused on the nitrogen isotope ratio of N_2O dissolved in water masses within and at the boundaries of the oxygen minimum zone.

Seawater samples were collected with 23-l Niskin bottles from various depths at six stations of the eastern tropical Pacific Ocean on legs 2 and 3 of KH-82-5 Cruise of RV *Hakuho Maru*, University of Tokyo²¹. Five seawater samples collected at 2-m depth intervals were successively and carefully introduced, without exposure to air, to a stainless steel tank which had been flushed and filled with argon. Dissolved N_2O was then extracted and collected on a molecular sieve 5A column using a device illustrated in Fig. 1. The nitrogen isotope ratio of the N_2O was determined later in the laboratory by a method described elsewhere¹⁷. The accuracy of the data presented here is estimated to be within $\pm 3\%$ for concentration and $\pm 0.2\%$ for $\delta^{15}\text{N}$ of N_2O both in the atmosphere and in the ocean.

The vertical profiles of N_2O were similar to those reported by other workers^{9,11–13,16}; N_2O increased with depth, attained a maximum and decreased at greater depths. Figure 2 depicts the relationship between dissolved oxygen concentration and dissolved N_2O concentration (top) or nitrogen isotope ratio in N_2O (bottom).

According to the nitrogen isotope ratio ($\delta^{15}\text{N}$ referred to atmospheric N_2 standard), dissolved N_2O can be categorized into two groups; one is depleted in ^{15}N (average $\delta^{15}\text{N} = 5.2 \pm 1.3\%$) relative to that in equilibrium with atmospheric N_2O (8.0% as indicated by the dotted line in Fig. 2), and the other is enriched in ^{15}N (average $\delta^{15}\text{N} = 12.6 \pm 1.1\%$). The former was found invariably at depths where the oxygen concentration was relatively high, while the latter occurred at depths where the oxygen concentration was extremely low and the N_2O concentration almost the same as or somewhat lower than the level in equilibrium with air estimated from the solubility data of N_2O (ref. 22). The high concentration of dissolved N_2O observed above the oxygen minimum layer can be attributed to the production of N_2O associated with nitrification^{18–20}. Our ^{15}N data support this inference, because we (N.Y. and S.M.,

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Table 1 Concentration and nitrogen isotope ratio of N_2O collected from maritime air over the Pacific Ocean on cruise KH-82-5 of RV *Hakuho Maru*

Sample no.	Location Latitude	Longitude	Station no.	Date (GMT)	N_2O (p.p.b.)	$\delta^{15}N$ (‰)
1	36°09' N	156°32' E	S4-5	25 November 1982	327	8.1
2	36°25' N	168°19' E	S8-9	27 November 1982	308	7.1
3	40°25' N	175°11' W	S14-15	29 November 1982	336	8.1
4	49°37' N	174°07' W	S14-15	29 November 1982	301	7.4
5	40°53' N	172°53' W	S14-15	29 November 1982	306	7.4
6	41°00' N	172°04' W	S15-16	29 November 1982	334	7.7
7	41°09' N	170°58' W	S15-16	29 November 1982	324	6.5
8	41°19' N	169°56' W	S15-16	30 November 1982	329	7.2
9	41°30' N	168°53' W	S16-17	30 November 1982	315	6.5
10	42°22' N	156°00' W	S20-21	2 December 1982	313	7.0
11	41°16' N	135°49' W	S26-27	5 December 1982	310	7.5
12	26°50' N	121°47' W	9	21 December 1982	327	3.7
14	17°15' N	108°38' W	13	26 December 1982	297	8.6
16	10°30' N	105°20' W	16	4 January 1983	287	7.9
19	2°29' S	115°05' W	19	8 January 1983	292	6.7
23	13°00' N	117°00' W	25	15 January 1983	305	6.6
Averages					313 ± 15*	7.2 ± 1.1

* As for the maritime air, Rasmussen and Pierotti²⁵ reported the N_2O content to be between 315 and 330 parts per 10^9 (p.p.b.). On the other hand, Weiss *et al.*²⁶ proposed that it was 302.8 ± 0.6 p.p.b. for this sampling time.

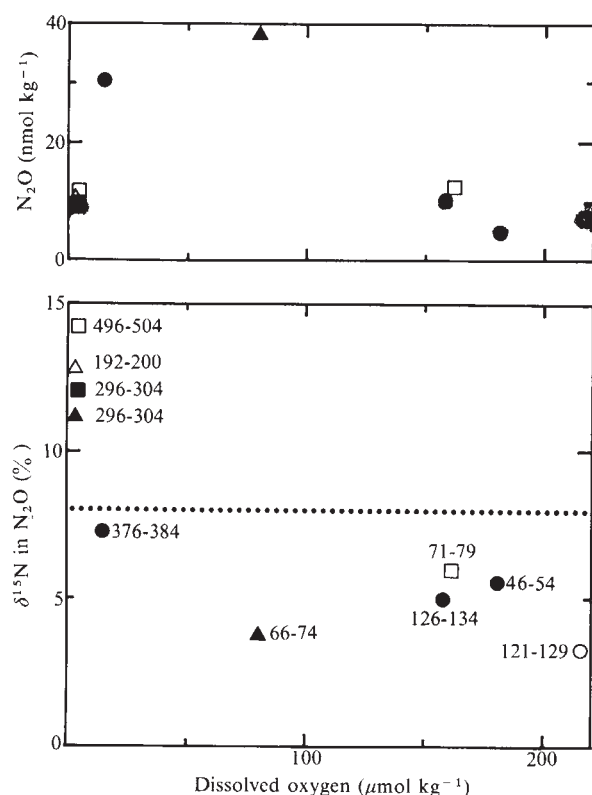


Fig. 2 Relationship between dissolved oxygen concentration and N_2O concentration (top) or nitrogen isotope ratio in N_2O (bottom) in the subsurface waters of the eastern tropical Pacific Ocean. Numerical values represent depth ranges where the water samples were collected. Symbols: $\circ$, station 9; $\triangle$, station 13; $\square$, station 16; $\bullet$, station 19; $\blacktriangle$, station 25; $\blacksquare$, station 11'. Sampling date and station location are given in Table 1 except for station 11' (18 January 1983, 17°27' N and 116°58' W). Oxygen data presented in the cruise report²¹ were used. A shaded bar (top) and a dotted line (bottom) refer to N_2O concentration and $\delta^{15}N$ in N_2O in equilibrium with atmospheric N_2O (averages in Table 1), respectively. The range of equilibrium concentration corresponds to the range of temperature and salinity of the seawater samples; from 8.58 °C and 34.62‰ to 28.21 °C and 35.04‰. The nitrogen isotope fractionation factor in the process of dissolution of N_2O in seawater is substituted for that in pure water which is 0.9992 (N.Y. and S.M., unpublished) in this temperature range; this means 0.8‰ enrichment of ^{15}N in the aqueous phase.

unpublished) have found a great depletion of ^{15}N down to -66‰ in the product N_2O relative to the initial reactant ammonium during nitrification by *Nitrosomonas europaea*. The lighter isotope was preferentially used to produce N_2O . Actually, excess N_2O (N_2O concentration observed - N_2O concentration in equilibrium with air at given temperature and salinity) was linearly correlated with AOU (apparent oxygen utilization) (data not shown), as observed by Yoshinari⁹, Elkins *et al.*¹¹ and Cohen and Gordon¹³.

The ^{15}N enrichment in N_2O in the oxygen minimum zone can also be explained by the isotope ratio of the nitrate source and by the isotope fractionation associated with denitrification. Cline and Kaplan²³ demonstrated that nitrate in the oxygen-deficient waters of the eastern tropical North Pacific is highly enriched in ^{15}N . N_2O enriched in ^{15}N may result from nitrate enriched in ^{15}N . Nitrous oxide enriched in ^{15}N may also be produced by the mode of reaction kinetics associated with denitrification, as observed in the case of nitrite by Blackmer and Bremner²⁴. ^{15}N enrichment in N_2O caused by isotope fractionation in reduction of N_2O is also likely. We (N.Y. and S.M., unpublished) have found nitrogen isotope fractionation factors of up to 1.027 in the reduction of N_2O by *Pseudomonas denitrificans*, where the lighter isotope was favoured in the N_2 produced. In fact, N_2O concentrations in extremely oxygen-depleted waters were far less than that expected from AOU.

The average nitrogen isotope ratio of N_2O in maritime air collected on the same cruise over the Pacific Ocean (Table 1) was 7.2 ± 1.1 ‰. This average was higher than that of dissolved N_2O in the subsurface layer above the oxygen minimum zone, but slightly lower than that of land surface air collected in Japan (8.1 ± 1.0 ‰)¹⁷. The observed difference suggests that the subsurface layer of the sea seems to emit ^{15}N -depleted N_2O and consequently lower ^{15}N content of N_2O in the maritime air. The results described here are still too premature to discuss this topic in further detail. However, we emphasize that nitrogen isotope studies have the potential to answer such questions.

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Rare earth element fluxes and geochemical budget in the eastern equatorial Pacific

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Rare earth element (REE) patterns and flux measurements of sediment traps, sediments, and nodules from the eastern tropical Pacific demonstrate the dominance of REE supply to the sea floor by particle settling. Quantitative partitioning of the particle flux reveals that biogenic debris is the major REE carrier in shallow depths; however, the labile particles are surpassed in importance by aluminosilicate particles at greater depths. Regeneration of biogenic-associated REE appears responsible for La/Ce variations in the particle flux with depth, and provides the primary source of REE accreted by nodules.

Studies of the fractionation of individual REE from one another have provided insight into the geochemical behaviour of REE in the marine environment¹⁻³. Ce, because of its unique preference for the 4+ state in seawater, may be more readily adsorbed onto water column particles⁴ and incorporated into deep-sea sediments and ferromanganese nodules⁵. Thus, the preferential adsorption of Ce may explain its depletion in seawater relative to the trivalent REE^{6,7}. Seawater is also enriched in the heavy REE (HREE), possibly as a result of the greater stability of aqueous complexes formed by HREE, leaving seawater relatively enriched in these elements^{6,7}.

Although previous studies have used REE fractionation to emphasize the importance of a particle-derived source of REE to sediments and nodules (ref. 2 and others therein), ours are the first reported measurements of the particulate REE flux. We present the REE contents, patterns and fluxes of sedimentary samples from the Manganese Nodule Program (MANOP) site H (6.5° N, 93° W) in the eastern equatorial Pacific in order to establish the REE geochemical budget for this site. In addition, we partition particulate REE into biogenic and detrital components. Decomposition of organic matter and dissolution of opal and carbonate can release REE which are sorbed to other particle surfaces or lost to seawater. Since such material decomposes more readily than detrital phases, the associated REE are more available for uptake by nodules and other authigenic phases.

Samples for this study were analysed for La, Ce, Nd, Sm, Eu, Tb, Yb and Lu by instrumental neutron activation⁸ (Table 1). Analytical precision for the REE was within 10% for all samples, except for Nd and Tb which have peak interferences. Interferences due to U were negligible.

Figure 1 shows the REE abundance patterns of site H samples normalized to that of world average shale⁵. Different sample types exhibit large variations in absolute concentration in the order: ferromanganese crust > nodule tops > sediment > nodule bottoms > sediment traps. Nonetheless, the general pattern for all samples is similar to that of site H bottom water (Fig. 1). All samples exhibit the Ce depletion and HREE enrichment typical of seawater. This does not, however, necessarily imply accretion by precipitation from seawater. Site H ferromanganese crust, dredged with basalts and isolated from sediment contact, is thought to form by direct precipitation from bottom water (hydrogenous accretion)⁹. This crust has the smallest Ce anomaly and least resembles the seawater pattern of all the site H samples (Fig. 1). In contrast, similar crusts of probable hydrogenous origin have large positive Ce anomalies¹⁰.

The REE patterns of site H nodules are unlike those of most deep-ocean nodules, which have positive Ce anomalies^{1,2}. Nodules from the Bauer Basin of the south equatorial Pacific, however, have negative Ce anomalies^{11,12} which are believed to result primarily from hydrothermal iron oxyhydroxides possessing a seawater-like REE pattern¹². The negative Ce anomalies of site H nodules may also indicate hydrothermal influence, since site H and the Bauer Basin are both within 1,000 km of the East Pacific Rise.

Accumulation rates (mass added per unit time per area) were calculated for site H crusts, nodule tops and bottoms, sediments, and trap material in order to provide a more complete understanding of REE cycling (Fig. 2). Distinctions between nodule tops and bottoms were based on texture and major element composition⁹. Results of site H flux calculations for Oregon State University single cone¹³ and Soutar double cone¹⁴ sediment traps deployed from September 1980 to October 1981 reveal increases in the total particle flux¹⁵ with depth. In addition, the increase in the particulate REE concentration with depth (Fig. 1) results in a substantial increase in REE flux with

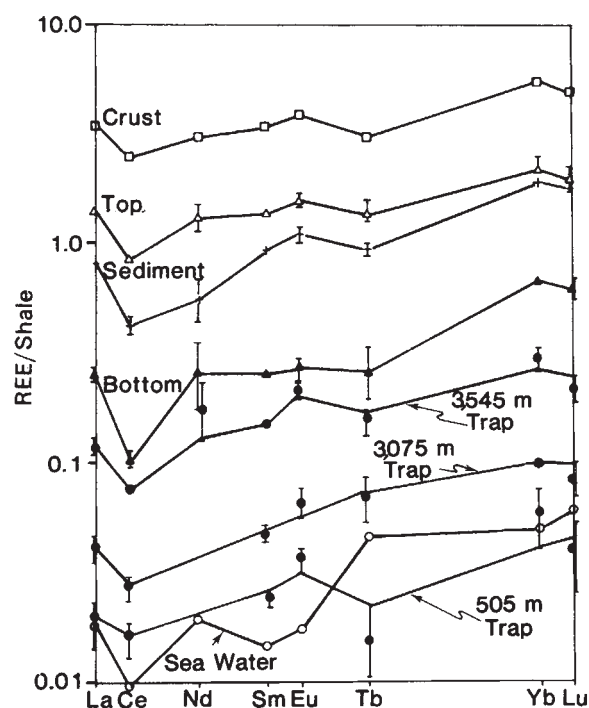


Fig. 1 REE abundances in ferromanganese crust, nodule top and bottom, sediment, sediment traps and bottom water normalized to the world average shale¹. Seawater data are for deep water from the Guatemala Basin near site H²⁰ and are multiplied by a factor of 10³. Values without error bars have errors within symbol size. Values not shown were below the detection limit or had associated errors >50%.

Table 1 Site H sample REE compositions (INAA data) (in p.p.m.)

Accession no.	Box core	La	Ce	Nd	Sm	Eu	Tb	Yb	Lu
Tops									
MP2827	I26-1	56.0	68.0	48.0	9.8	2.43	1.61	7.4	1.14
MP2880	I26-4	48.0	52.0	42.0	9.5	2.22	1.55	6.9	1.05
MP2830	I27-1	35.0	37.0	29.0	6.8	1.71	1.13	6.0	0.92
MP2833	I27-3	16.0	10.0	14.0	3.0	0.76	0.60	2.9	0.47
MP2835	I34-1	29.0	40.0	26.0	5.6	1.41	0.87	5.0	0.76
MP2836	I34-2	35.0	42.0	32.0	7.1	1.67	1.19	5.4	0.82
MP2886	I34-4	22.0	17.0	22.0	4.4	1.20	0.78	3.7	0.55
MP2840	I36-1	34.0	37.0	30.0	6.3	1.58	1.01	5.4	0.82
MP2842	I36-2	46.0	60.0	35.0	7.9	2.17	1.30	6.3	0.93
MP4837	V37-1	46.0	58.0	40.9	8.9	2.12	1.51	7.3	1.04
MP5344	V52-1	33.0	48.0	35.0	6.8	2.09	1.01	5.9	0.82
Bottoms									
MP2826	I26-1	10.0	10.0	11.0	2.1	0.43	0.26	1.7	0.28
MP2828	I26-2	14.0	8.0	13.0	2.6	0.57	0.43	2.3	0.36
MP2879	I26-4	11.0	10.0	10.0	2.1	0.43	0.19	1.7	0.26
MP2829	I27-1	9.0	11.0	9.0	2.1	0.40	0.37	1.7	0.25
MP2832	I27-3	10.0	13.0	10.0	2.0	0.44	0.21	1.6	0.24
MP2882	I27-4	11.0	11.0	9.0	2.0	0.45	0.37	1.8	0.30
MP2834	I34-1	12.0	11.0	10.0	2.2	0.51	0.45	2.1	0.30
MP2837	I34-2	13.0	10.0	8.0	2.6	0.64	0.49	2.4	0.36
MP2885	I34-4	10.0	9.0	11.0	2.2	0.49	0.41	1.8	0.27
MP2839	I36-1	15.0	11.0	12.0	2.5	0.60	0.46	2.5	0.39
MP2841	I36-2	16.0	16.0	12.0	2.7	0.68	0.51	2.8	0.43
MP4836	V37-1	11.0	14.0	10.0	1.8	0.50	0.28	2.3	0.46
MP5345	V52-1	8.0	12.0	9.0	1.4	0.47	0.35	1.5	0.24
Whole nodules									
MP2881	I26-4	18.0	18.0	15.0	3.3	0.77	0.54	2.9	0.45
MP2831	I27-2	35.0	38.0	31.0	6.9	1.70	1.27	6.0	0.92
MP2883	I27-4	12.0	12.0	11.0	2.3	0.54	0.41	2.2	0.34
MP2838	I34-3	21.0	16.0	21.0	4.1	1.10	0.85	3.8	0.58
MP2884	I34-4	20.0	17.0	17.0	3.9	0.95	0.55	3.3	0.52
MP2887	I36-4	17.0	17.0	19.0	3.3	0.85	0.58	3.0	0.47
Crusts									
MP9843	P11-4	136.0	196.0	114.0	25.0	6.10	3.68	19.0	2.89
MP9844	P11-5	131.0	210.0	109.0	23.0	6.22	3.55	18.0	2.64
Sediments									
MP5301	BC37-1	33.0	35.0	29.0	7.0	1.8	1.3	6.3	1.1
MP5302	BC37-2	32.0	34.0	20.0	6.9	1.6	1.1	6.4	1.1
MP5304	BC37-4	38.0	38.0	44.0	7.2	1.8	1.3	6.8	1.1
Traps									
MPT9640	H1-U	0.83	1.4	—	0.16	0.059	—	0.22	0.031
MPT9525	H1-S	1.18	2.1	—	0.26	0.099	0.049	0.30	0.042
MPT9797	H1-L	1.66	2.1	3.8	0.35	0.10	0.084	0.32	0.049
MPT9527	H2-U	2.16	2.8	5.8	0.44	0.13	0.076	0.48	0.069
MPT9530	H2-S	2.77	3.5	2.5	0.63	0.17	0.11	0.45	0.076
MPT9531	H2-L	4.91	6.2	6.3	1.09	0.33	0.19	0.97	0.15

depth (Fig. 2). This may be caused by adsorption of REE onto particulate debris as it falls through the water column and/or additional sources of REE-rich particles at depth. Within the upper 1,500 m of the water column, there is a substantial increase in REE particulate flux with depth. Such an increase has been noted in other oceanic locations and may be due to lateral transport of material¹⁶, particle aggregation¹⁷, or zooplankton feeding dynamics¹⁸. Below 1,500 m REE flux increase is gradual until within approximately 500 m of the bottom. Below this, there is a sharp increase in apparent flux which is due to resuspension of bottom material¹³. Therefore, in calculating sediment trap REE fluxes to the sediment-water interface, an extrapolation of fluxes between 1,465 m and 3,075 m depths was used to avoid the influence of local resuspension which produces an artificial flux increase in near-bottom traps.

Comparison of the extrapolated trap flux to the elemental accumulation rate in the sediment reveals that the total REE particulate flux accounts for approximately 85–100% of the REE preserved in site H sediments (Fig. 2). Hydrogenous precipitation, presumably represented by accretion in ferromanganese crust, is another potential source of REE to the

sediments. However, since REE accumulation in crust accounts for less than 7% and 1% of the REE accumulation rates in nodules and sediments, respectively (Fig. 2), the contribution from the hydrogenous source appears relatively insignificant, and particulate debris is apparently the major source of REE to site H nodules and sediments. Near-bottom enrichments of REE in the North Atlantic³ suggest that REE are released by regeneration of labile biogenic particles at the sediment-water interface¹⁷. However, our geochemical balance (Fig. 2) implies that REE at H are strongly retained by sedimentary phases. Nonetheless, the range in bulk sedimentation rate (Fig. 2), the resuspension assumptions of the flux calculations, and uncertainties involved in comparing a single year of trap data with the sediment record, limit the precision of the extrapolated and observed flux comparison. For several refractory elements, such as Al, the agreement between sediment accumulation and extrapolated particle flux is within 10%, while labile elements, such as Cu, show 40–50% regeneration at the sediment-water interface¹⁵. Results for the REE clearly show that a particulate source is the major supply to site H sediments and that the fraction of REE regenerated from particles at the sea floor is small.

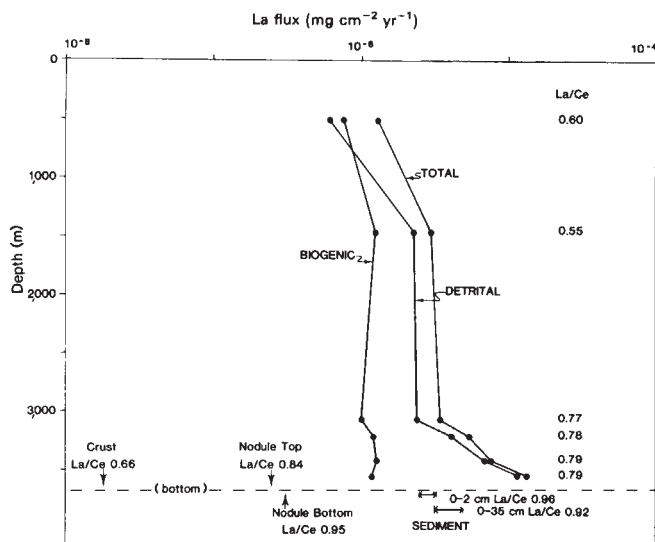


Fig. 2 La fluxes and La/Ce ratios of site H samples. Regression-derived REE concentrations and ratios: (1) biogenic: La = 0.5 p.p.m., Ce = 1.0 p.p.m., La/Ce = 0.50 ($r^2 = 0.99$); (2) detrital: La = 40.5 p.p.m., Ce = 43.0 p.p.m., La/Ce = 0.93 ($r^2 = 0.98$). Accumulation rates of REE in crusts, nodule tops, nodule bottoms, sediments and sediment traps were calculated using $A = SC\rho$; where A is the flux or accumulation rate of an element in a sample; S is the bulk growth rate, sedimentation rate, or flux applying to crusts/nodules, sediments and traps, respectively; C is the concentration of an element in a sample; and ρ is the dry bulk density of nodules, crusts or sediments. Biogenic and detrital fluxes were derived from linear programming. Crust growth rates are estimated at 1 mm per 10^3 yr (ref. 25). Nodule top/bottom growth rates were determined by refs 26 and 27. Sediment accumulation rate ranges are based on sedimentation rates of 0.45 cm per 10^3 yr (ref. 28) and 0.65 cm per 10^3 yr (ref. 29). Dry bulk density values of 1.35 g cm^{-3} (ref. 24) and 0.22 g cm^{-3} (K.M., unpublished data) were used for crust/nodules and sediment, respectively.

To clarify further the REE budget at site H, sediment trap and surface sediment samples were partitioned into end-member components using normative analysis. Weight fractions of carbonate, opal, organic matter and ferromanganese precipitates, which best account for the abundance of 13 elements (organic C, P, N, Al, Si, Ca, Ti, Mn, Fe, Cu, Ni, Zn and Co) in 27 annual and seasonal trap and surface sediment samples from MANOP sites H and M (9°N , 104°W), were estimated by linear programming¹⁹. The carbonate, opal and organic matter fractions were combined as 'biogenic material'. Regression lines were determined between the measured REE content and the percentage of biogenic or detrital component. Extrapolation of the line to 100% of each component allows estimation of the concentrations of pure end-members (Fig. 2). The REE flux from each source can be computed from the end-member fractions in each sample and the regression-derived REE end-member concentrations (Fig. 2). The model, of course, assumes biogenic and detrital components have a constant composition. Since the REE content of particles may result from scavenging, compositional variability with depth may occur which would add uncertainty to the normative analysis.

Table 2 shows that the sum of the regression-derived biogenic and detrital fluxes approximates the measured La flux within 10%, for five out of six traps. The biogenic and detrital fluxes account for nearly all the REE flux throughout the water column, while the ferromanganese end-member, possibly representing hydrothermal influence, is relatively insignificant in all traps (K.M. and J.D., unpublished data). The results demonstrate that the biogenic REE flux is dominant in the upper 750 m of the water column but is surpassed by the detrital flux below this depth (Table 2, Fig. 2). Within the bottom 500 m, resuspension of detrital material, which is relatively enriched in the

Table 2 Percentage of total measured La flux explained by end-members in annual traps

Trap depth (m)	Biogenic (%)	Detrital (%)	Difference between measured and calculated flux* (%)
505	60	48	8
1,465	41	76	17
3,075	29	70	1
3,225†	22	75	3
3,415†	17	91	8
3,545†	9	86	5

* Calculated flux = regression-derived biogenic + detrital fluxes.

† Resuspension influenced¹³.

sediments, distorts the true flux of the two components. Nevertheless, the detrital REE flux increases gradually with depth, while the biogenic REE flux decreases slightly. This trend probably results from the decomposition and dissolution of biogenic debris as it falls through the water column, combined with an increase in the detrital fraction due to resuspension at depth. The observation that the biogenic REE flux comprises 30–60% of the total flux to site H sediment traps not influenced by resuspension (Table 2) is consistent with the suggestion that REE are associated with biogenic particles³.

The La/Ce values suggest that some fractionation occurs between Ce and the trivalent REE. This ratio is a more direct means of comparison and has greater precision than the Ce anomaly, particularly for sediment traps which have low REE abundances and greater analytical errors for Nd (Fig. 1). The biogenic component of trap debris, with a La/Ce ratio of 0.50, is enriched in Ce relative to the detrital end-member, with a La/Ce of 0.93 (Fig. 2). Both components, however, are enriched in Ce relative to La, and although deep-ocean nodules have been claimed to be responsible for the REE pattern of seawater¹, a quantitative accounting of the Ce enrichments in these phases could also explain the negative Ce anomaly of seawater. The La/Ce ratio of Guatemala Basin bottom water near site H is approximately 2.0 (ref. 20), and North Atlantic waters below 200 m also have La/Ce ratios ranging between 1.0 and 2.4 (ref. 3). Therefore, the biogenic component at site H is enriched in Ce relative to La when compared with seawater. Elderfield and Greaves' study in the North Atlantic³ revealed surface water enrichments of Ce that were high compared with the trivalent REE, presumably due to preferential diffusion of Ce from reducing continental margin sediments²¹ and advection of margin waters. Since site H could also receive excess Ce by advection from the slope region, the extent of La/Ce fractionation by biogenous debris is uncertain without surface water REE data.

We find a consistently lower La/Ce ratio for traps from the upper 1,500 m (La/Ce $\approx$ 0.6) compared with La/Ce values of the near-bottom traps (La/Ce $>$ 0.75) (Fig. 2). This trend may result from the aforementioned input of Ce mobilized from near-shore anoxic sediments. It is hypothesized that Ce, like Mn (refs 22, 23), is solubilized at locations where the oxygen minimum intersects the continental margin. The unusually high Mn content of site H trap particles¹⁵ implies that near-shore reduction and subsequent remobilization of Mn influence the composition of particles at this location. Alternatively, the La/Ce increase with depth may be due to decomposition of Ce-enriched labile biogenic particles that release REE to seawater and increase the influence of the detrital fraction which is relatively depleted in Ce. Trapping of resuspended sediments in the deepest traps would also result in higher La/Ce, since the La/Ce of sediments is about 1.0. The 3,075 m trap, which is more than 500 m above the bottom, is believed to be free of resuspended material, however²⁴. Since it, too, has elevated La/Ce compared with the near-surface traps, it appears that

resuspension is not the sole explanation for the La/Ce increase with depth. Moreover, the La/Ce of the deepest traps (0.8) does not match the La/Ce ratio of site H sediments (1.0). Fractionation of the REE, which apparently occurs during sediment diagenesis, may explain the increased La/Ce of site H sediments. During periodic, highly reducing conditions, Mn is remobilized within the sediments and conveyed to nodule bottoms⁹. Under such conditions, Ce may be reduced to its trivalent state and mobilized to nodules, and possibly into the bottom water²¹. Although the trivalent REE should also be mobilized under these conditions, enhanced Ce release could result if the Mn-rich phase that dissolves is also enriched in Ce relative to the other REE.

Thus, sediment trap material, sediments and nodules from the eastern tropical Pacific all have seawater-like REE patterns. Flux calculations imply that particles can account for most, if not all, of the REE supply to site H sediments and can easily account for REE accumulation rates in nodules. Breakdown of

the particulate flux into end-member components discloses the importance of biogenic material as well as detrital particles in conveying REE to the ocean bottom at site H. The La/Ce ratios of the biogenic and detrital trap components and traps throughout the water column confirm the importance of both components in supplying REE to sediments and nodules and indicate the release of REE from labile biogenic components. Although biogenic and detrital material both contribute REE to sediment traps, it is the biogenic fraction which undergoes decomposition and dissolution reactions, that probably conveys the majority of REE to nodules.

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Apparent changes in the climatic state of the deep North Atlantic Ocean

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Determination of any long-term changes in the large-scale characteristics of the deep ocean circulation would be an important clue in understanding the climatic interactions of the ocean and atmosphere. In the summer of 1981, the RV *Atlantis II* reoccupied two transatlantic sections at nominal latitudes of 24°30' N and 36°16' N with a conductivity-temperature-depth instrument (CTD). One purpose of the work was to make a comparison with previous surveys conducted during the International Geophysical Year (IGY)¹, when sections were obtained in October 1957 and April-May 1959. We report here that significant warming occurred in an ocean-wide band from 700 m to 3,000 m with a maximum temperature difference of 0.2 °C. These changes are sufficient to expand the water column by several centimetres. The historical temperature-salinity curve was apparently unchanged. Interannual changes in local water mass characteristics have been proposed previously^{2,3}. Perhaps it would be most surprising if no changes were seen to occur. What remains obscure is the significance of these changes and the extent to which they represent long-term climate trends, or merely the minor and random fluctuations to be expected in any complex fluid system.

To make a direct comparison of temperature in the two investigations, we have interpolated the data to common locations (excluding segments at the ends where the lines did not coincide). A fine mesh was generated (grid points over 40 m vertically and every 10 km horizontally) using the technique of objective mapping^{4,5}. Subtraction of IGY temperatures from the 1981 temperatures gave temperature differences, but maps of temperature difference have strong mesoscale features that make it difficult to pick out large-scale trends. To suppress the small scales, a horizontal gaussian filter 1,000 km wide was applied to produce smoothed maps of temperature difference (Fig. 1a (24° N) and b (36° N)).

The most prominent feature of the temperature difference maps is an ocean-wide band of warmer water at middle depths of both latitudes. This band is much thicker and more intense in the western basin, where it extends from a depth of a few hundred metres to greater than 3,500 m. Relative to the IGY survey, mid-depth isotherms in the 1981 survey were lower overall and were tipped downward towards the west. Other strong features are seen in Fig. 1, such as the large area of negative temperature difference at 36° N, centred at about 500 m, just west of the mid-Atlantic ridge. However, these other features are not clearly similar at the two latitudes (they appear in different temperature ranges) and therefore cannot be said to be of ocean scale. Features high in the water column are likely to result from local processes while characteristics of the deep water are determined at remote source regions at high latitudes⁶.

Figure 2 shows temperature difference averaged across the width of the ocean. The average warming begins at a depth of 500-700 m and extends to 3,000 m with a peak of about 0.2 °C at 1,000-1,500 m. By computing an average vertical temperature gradient, temperature difference was converted to an equivalent vertical displacement of isotherms (the vertical temperature gradient is much greater at 1,000 m than at 2,000 m). Vertical displacements were approximately 20 m and 100 m

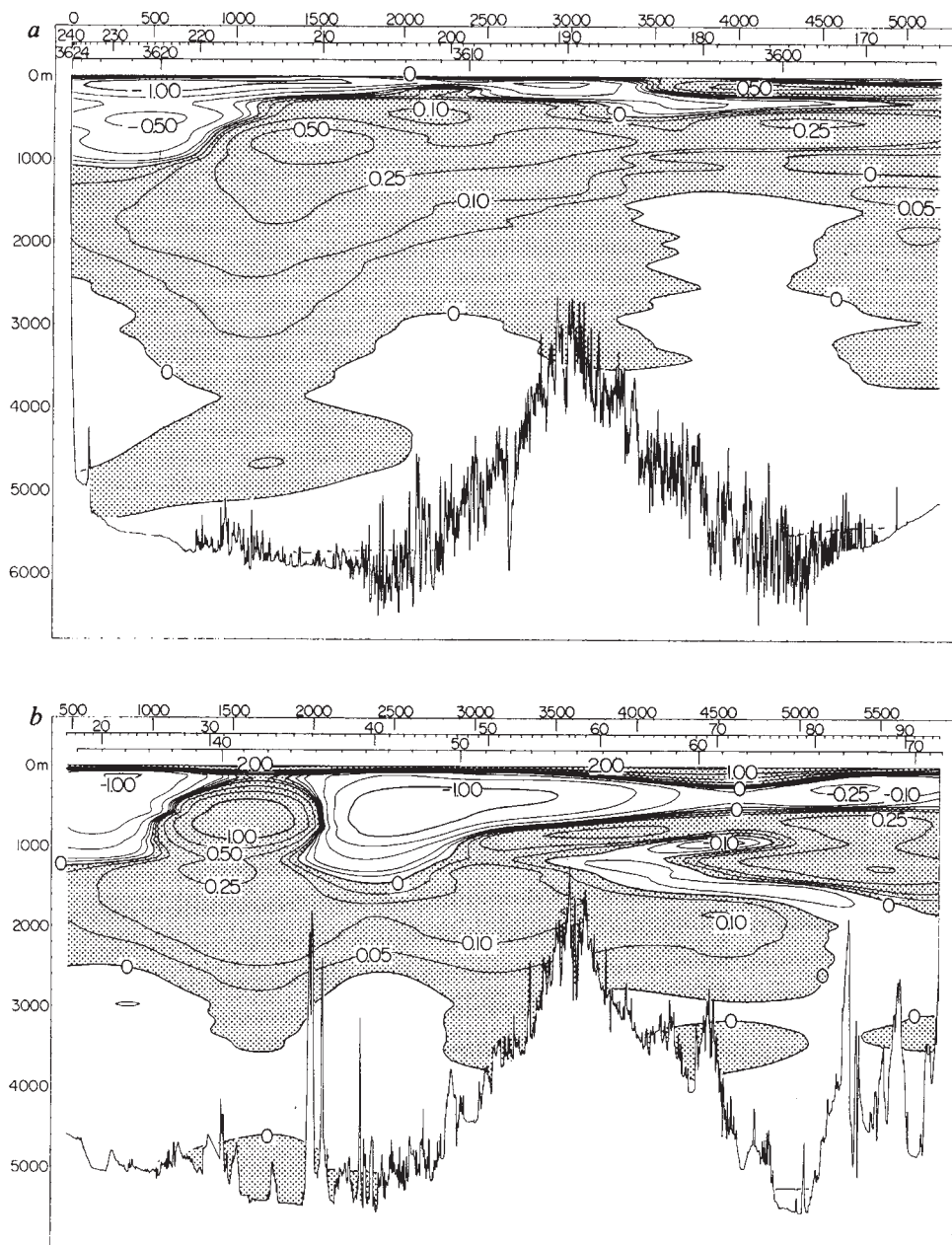


Fig. 1 *a*, Smoothed temperature difference section; IGY data subtracted from 1981 values, at 24° N. Distance scale at top in km, middle and bottom scales show 1981 and IGY station numbers respectively. *b*, Smoothed temperature difference, IGY data subtracted from 1981 values, at 36° N.

downward at 1,000 and 2,000 m respectively. The displacement at 2,000 m is large enough to be seen in a visual comparison of temperature maps from the 1981 and IGY sections, noting, for example, a drop of about 100 m in the 4 °C isotherm. Below 3,000 m, average temperature differences were small but tended to be negative. Above 700 m, a strong but thin band of negative difference is seen at 24° N at about 200 m and at 36° N a thicker band extends from 100 to 700 m. This upper-ocean cooling trend has been noted previously⁷ at the Panuliris station (32° N, 64° W). At 24° N, the positive temperature difference at mid-depth dominates the vertical profile. Excluding the upper 100 m, where seasonal effects are important, the 1981 section at 24° N is 0.03 °C warmer than the IGY section. At 36° N, the positive differences are balanced by the negative ones and the total average temperature is nearly unchanged.

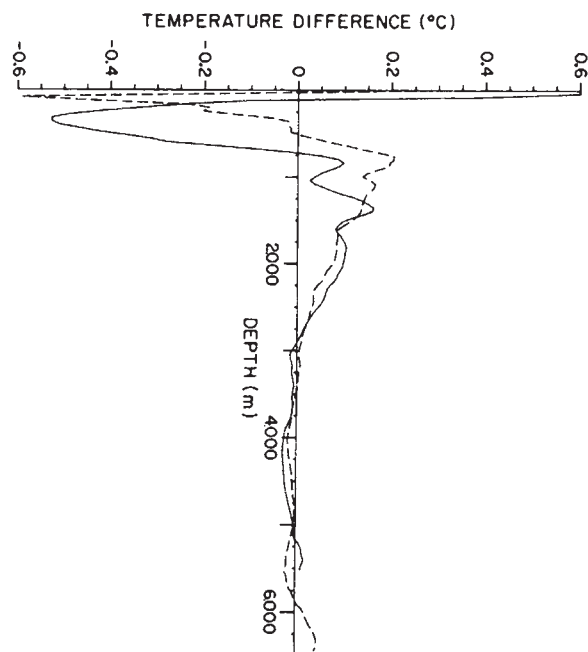
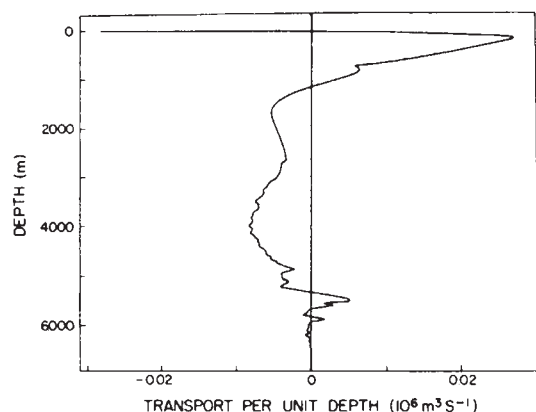
The tendency in salinity changes (not shown) was to maintain the historical temperature/salinity correlation; that is, positive temperature differences were usually accompanied by positive salinity differences. Because we observe greater vertical displacements in isotherms at some levels than at others, changes in the volumes occupied by different water masses must have occurred.

To study the expansion and contraction of water masses, the ocean was divided into six layers separated by surfaces of constant potential temperature. The six layers, chosen on the basis of earlier work⁸, are shown in Table 1 with the area occupied by each of these water masses in each of the four sections. At both 24° N and 36° N there is an expansion in bottom water (<2 °C), a contraction in deep water (2–4 °C) and an expansion in middle and lower thermocline water (4–12 °C). Summing the change in deep and bottom water, the net contraction of water cooler than 4 °C lowers the isotherms of the middle and lower thermocline, consistent with the observation of warmer water at mid-depth.

In a discussion of error sources in the preceding calculations, measurement and mapping errors (negligible for present purposes) are included, as well as errors caused by the presence of short time scale eddies. The latter were found to be the largest error source, with a single intense eddy able to perturb the 1,000 m temperature by about 2 °C over 200 km, while the perturbation at 2,000 m is about 0.3 °C. Averaged over a 5,000 km basin, these estimates result in a bias of 0.08 °C at 1,000 m and 0.01 °C at 2,000 m. Because we observed the same pattern of change at both 24° and 36° N, and because the 0.1 °C

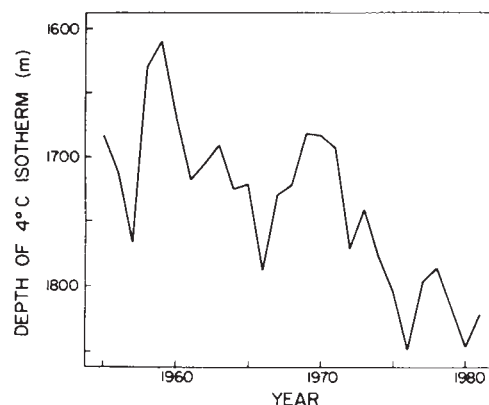
Table 1 Water mass census

Potential temperature class (°C)	Area (km ²) 24° N 1957	Area (km ²) 24° N 1981	Change 1981-1957	% Change	Area (km ²) 36° N 1959	Area (km ²) 36° N 1981	Change 1981-59	% Change
>17	1,751	1,753	2	0.1	936	998	62	6.6
12-17	1,451	1,502	51	3.5	2,124	1,890	-234	-11.0
7-12	1,716	1,778	62	3.6	3,108	3,277	169	5.4
4-7	4,008	4,238	230	5.7	3,413	3,748	335	9.8
2-4	12,795	12,107	-788	-6.2	11,425	10,870	-555	-4.9
<2	5,604	5,961	357	6.4	2,242	2,465	223	9.9

**Fig. 2** Temperature difference, IGY data subtracted from 1981 values, averaged over the width of the ocean. Solid line, 36° N; dashed line, 24° N.**Fig. 3** Northward geostrophic transport per unit depth across 24° N (including the Straits of Florida).

signal at 2,000 m is much greater than the noise level, we see that the signal is not due to eddy noise. The only other error potentially of concern is in the depth inference; the IGY data depths are based upon reversing thermometers and those for the 1981 data upon a pressure sensor. The expected discrepancies can be shown to be less than about 10 m, well below the observed displacements.

The hydrographic station data can also be used to calculate geostrophic velocities and volume transports. Figure 3 shows horizontally-integrated transport per unit depth across 24° N (1981 data). Geostrophic shear together with mass conservation

**Fig. 4** Annual mean depth of 4° C isotherm at the RV *Panuliris* station (32°10' N, 64°30' W).

constraints were used to estimate geostrophic velocity using an inverse method⁹. On the whole, surface and intermediate waters flow northward. Deep water flows southward in two lobes from its source regions in the Labrador and Greenland Seas and there is a small northward flow at the bottom. An interesting question is whether the observed water-mass shifts can be seen as the divergence of mass and/or heat transports. If the observed loss of mass in the 2-4 °C range was spread out evenly over the 23 yr between surveys, then a mass flux divergence of about $2 \times 10^9 \text{ kg s}^{-1}$ would be required. Relative to the transports of Fig. 3, which are of the order of $10^{10} \text{ kg s}^{-1}$, this value is too small to be detected reliably in geostrophic calculations. Similarly, the heat flux convergence required to add 0.1 °C to a layer 2,000 m thick is about $1 \times 10^{13} \text{ W}$, which is only about 1% of the total northward heat flux across 24° N.

What is the proper time scale of the hydrographic changes we have described? Some deep water masses are thought to form only sporadically³, with new formation being absent for years or longer. Are the differences we observe due to a 'sloshing' in the North Atlantic, measured in years, or are they part of a long term trend of expansion and contraction of water masses?

A simple north-south displacement of isotherms can be ruled out as the cause of the observed changes. The 4 °C isotherm depth is near its maximum value at 24° N. One can see this from meridional sections of temperature along 50° W and 66° W¹. No north-south displacement could increase the 4 °C depth by 100 m, as was observed in 1981.

Unfortunately, there are very few long time-series of measurements with which to address the time scale problem. The *Panuliris* station has been occupied regularly to depths of about 2,000 m since 1955. We analysed these data by interpolating each station to locate the depth of the 4 °C isotherm and then averaging all stations in a year. The 4 °C isotherm was chosen because of its large observed displacement in our study. The annual means of 4 °C isotherm depth (Fig. 4) show a clear trend, increasing by the order of 100 m over 25 yr. This evidence supports the hypothesis of a long time scale in our observations. At temperatures warmer than 4 °C, up to the waters where a long-term cooling has been previously described⁷, any possible

trends were obscured by eddy noise, which is large in this part of the Atlantic.

The present study is limited to the subtropical North Atlantic Ocean. Nevertheless, the questions that it raises are global. (1) Is the average temperature of the oceans increasing? Climate models which couple an atmosphere with a stratified ocean¹⁰ show ocean warming in response to the global increase of atmospheric carbon dioxide, but warming of the model oceans is concentrated near the surface, in contrast to our results. (2) Can the global rise in sea level, reported to be 10 cm per century¹¹, be attributed to thermal expansion of the deep ocean? The thermal expansion coefficient of seawater is about 1.6×10^{-4} per °C at 5 °C, 35‰ in salinity, and 200 bar¹². Thus, a column of water 1,000 m thick, heated by 0.1 °C gains nearly 2 cm. In the 1981 investigation, the water from 70 to 300 bar was thicker than in the IGY data by an average of 3 cm at 24° N and 2 cm at 36° N. Whether such changes occurred elsewhere in the world oceans is unknown.

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Suspended particulate organic material from hydrothermal vent waters at 21° N

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Ocean floor spreading centres described recently consist of several sites with chemical and biological features unlike any oceanic sites previously known^{1–4}. The high-pressure and warm-temperature regimes at these sites, coupled with dynamic hydrothermal water circulation, support biological communities that utilize a source of primary production which appears to be non-photosynthetic⁵. A detailed biochemical analysis of the suspended matter at a biologically-active site has been undertaken, with the goal of describing the relationship between microorganisms, benthic organisms and suspended particulate material within these hydrothermal systems. Suspended particulate matter from warm hydrothermal vent waters (5–38 °C) of the East Pacific Rise^{1–4} (21° N, 106° W) was sampled on the OASIS expedition with an *in situ* pumping system in April

1982. Particulate organic carbon (POC) and nitrogen (PON) as well as individual organic compound classes from the lipid fraction were analysed to ascertain the sources and composition of the organic matter in this highly productive area. We show here that the particulate organic material (POM) of the vent water was both quantitatively and qualitatively different from the non-vent bottom water. The POC and PON measured within the vent and non-vent waters vary by a factor of 2–5, the higher values being in warm water. The organic matter in the non-vent water was found to be more resistant towards degradation or remineralization than in the vent water, as shown by the C:N ratio and the total lipid:POC ratio. Contributions to the lipid class compounds were assessed using well-known organic compound source biomarkers such as hydrocarbons, fatty alcohols, sterols, triacylglycerols and steryl and wax esters.

The hydrothermal vent sampling site was ~50 m², located adjacent to a collapsed lava lake. Twin peaks of 'black smoker' vents ($T \sim 270$ °C), a large 'white smoker' vent ($T \sim 10$ –44 °C), and several other features were located in this area. Suspended particulate material from this site was sampled with an *in situ* pumping system. Four of the six filters were direct samples of warm (15–40 °C) hydrothermal vent water, and were from the following sites: (1) two from a hole located in the pillow basalt (dives 1212 and 1214) and (2) two from the base of a dense growth of vestimentiferan worms (*Riftia*) located adjacent to the white smoker (dives 1223 and 1220). One sample was of water intermediate in location and temperature between the warm vent water and non-vent bottom water (5 °C, above a bed of *Calymene magnifica*, dive 1212). The final sample was of non-vent bottom water at the vent site (2 °C, dive 1228). This sample will be referred to as the non-vent water sample. The *in situ* pumping system used to sample the particulate material consisted of a peristaltic pump, three solenoid valves, a polyethylene water bag, a five-in-one filter holder, a stainless steel single filter holder, and a computerized controller with associated electronics and power supplies. The pumping system was deployed from DSRV *Alvin* and sampled the hydrothermal vent water by directing the nozzle into a warm water source. Temperature readings of the water were made using a thermistor probe on *Alvin*. The vent water was pumped through Gelman type A/E glass fibre filters (GFF) at a rate of 1.6 l min^{-1} . The average sample size was ~150 l. The filters were previously combusted at 450 °C and Soxhlet extracted with 1:1 v/v toluene/methanol (MeOH) for 12 h. These filters retain >95% of particles of >1.0 µm diameter⁶. As previous studies indicate

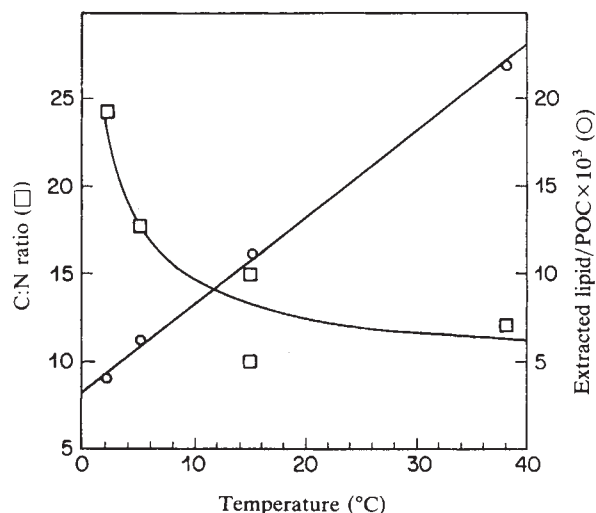


Fig. 1 The ratio of extracted lipid from hydrothermal vent water particulate material ($\mu\text{g l}^{-1}$) to the particulate organic carbon ($\mu\text{g l}^{-1}$) from the same sample, plotted as a function of water temperature, and the ratio of particulate organic carbon ($\mu\text{g l}^{-1}$) to particulate organic nitrogen ($\mu\text{g l}^{-1}$) plotted as a function of water temperature.

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Table 1 *In situ* pump sample description and bulk chemical properties

Dive no.	1228	1221	1212	1214	1223	1220
Description site	Pillow basalt	Clam bed	Hole in basalt	Hole in basalt	Base of white smoker	Base of white smoker
Water temperature (°C)	2	5	12–20	12–20	~38	—
Volume filtered (l)	160	164	—	157	158	34
POC ($\mu\text{g C l}^{-1}$)	91.1	139	194	218	163	219
PON ($\mu\text{g N l}^{-1}$)	4.4	9.2	15	25	16	22
C:N (atom)*	24.2	17.6	15.1	10.2	11.9	11.6
Total lipid extract (TLE) ($\mu\text{g l}^{-1}$)	0.37	0.85	—	2.4	3.6	11.5
TLE/POC $\times 10^3$	4.1	6.1	—	11.1	22.1	52.5
Filter description	Few macroscopic particles	Some macroscopic particles	—	24 copepods, few particles	30 large amphipods, many particles	Densely covered with inorganic sulphide particles

* C/N (weight) 1.167 = C/N (atom).

that >60% of the ATP biomass resides on particles >12 μm in hydrothermal vent waters⁵, the filters should sample a large fraction of the particulate organic material from this source.

Particulate organic carbon and nitrogen were analysed by filtering several litres of the vent water (collected in polyethylene bags) through a stainless steel filter holder containing two 25-mm Gelman type A/E GFFs which had been previously combusted at 500°C. The POC and PON determinations were accomplished using a CHN analyser.

The particulate material was also analysed for lipid class compounds using methods previously described⁷. The filters were weighed and ~25% of each was sonicated and extracted with methylene chloride (CH_2Cl_2)/MeOH/ H_2O . The lipid extract was then partitioned between CH_2Cl_2 and H_2O . The CH_2Cl_2 extract was evaporated to dryness under vacuum at <25°C. Hexane was then added and elemental sulphur was removed on a short column of activated copper particles (105–250 μm diameter)⁸. The sulphur-free extracts were separated into individual compound classes by adsorption chromatography. Elution with a series of solvents of increasing polarity separated the lipids into alkane and alkene, polyunsaturated alkene, sterol and wax ester, triacylglycerol, fatty alcohol, and steroid alcohol fractions. The alcohols were converted to their respective acetates with acetic anhydride in pyridine. The individual fractions were spiked with appropriate external standards for quantitative determinations and analysed by high resolution glass capillary gas chromatography (GC) using a deactivated, cross-linked SE-52 coated (25.5 m $\times$ 0.32 mm i.d.) column. Compound structures were identified by comparison of GC retention times (alkanes, wax esters, and triacylglycerols) and mass spectra with authentic standards (fatty alcohols and steroid alcohols)⁹. Procedural blanks performed concurrently with these analyses indicated <1% contamination in each fraction.

The bulk chemical properties from the *in situ* pump samples are listed in Table 1. Water temperature roughly increased from the pillow basalt sample (dive 1228) of 2°C non-vent water at the vent site, to 5°C for the sample collected above the clam bed (dive 1221), to 15–38°C for the directly sampled warm vent waters. The concentration of both POC and PON increased with increasing water temperature, but not in the same proportion. The total lipid extract (TLE) also increased with increasing temperature (Table 1). Similarly, the ratio of (TLE:POC) $\times 10^3$ ranged from a value of 4 in the non-vent bottom water to values of 11–50 in the hydrothermal vent waters (Fig. 1). A comparison of these POC and PON values with other values found in the world's ocean is informative. Western North Atlantic surface coastal POC values are ~100 $\mu\text{g l}^{-1}$, whereas open ocean POC values are ~50 $\mu\text{g l}^{-1}$ (ref. 9). Wangersky found POC values for surface waters (0–100 m) of 54 $\mu\text{g l}^{-1}$ for the South Atlantic and 30 $\mu\text{g l}^{-1}$ for the Pacific Ocean¹⁰. At depths >3,000 m the

POC value was ~5 $\mu\text{g l}^{-1}$ (refs 10, 11) for both the South Atlantic and Pacific Oceans. In the central North Pacific gyre, POC values range from 2 to 4 $\mu\text{g l}^{-1}$ (ref. 11). Off the coast of Peru, a productive upwelling site, POC values range from 100 to 700 $\mu\text{g l}^{-1}$ in surface waters¹². The values for warm vent water POC are in the region of 200 $\mu\text{g l}^{-1}$ indicative of a relatively productive site.

The C:N ratio ranged from a value of 24.3 for non-vent bottom water to values of 10–15 in the warm vent water samples (Fig. 1). The values for the vent water C:N ratio are higher than expected for normal marine bacteria, or phytoplankton (~5–6)¹³, but similar to deep sea suspended POM. The C:N ratio for hydrothermal vent organisms, however, is not known. Values for dissolved organic carbon (DOC) for the warm vent water samples range from 0.64 to 0.85 mg C l^{-1} , somewhat lower than non-vent water (1.01 mg C l^{-1}).

Each filter analysed for lipids was qualitatively different. In general, the samples collected from lower temperature water contained less macroscopic particulate material than samples from higher temperature water. The three warm vent water filters were each qualitatively different from one another. Two had numerous large zooplankton, copepods (Spinocalanidae, new genus and species¹⁴) on filter 1214, and amphipods (genus *Onesimus* sp. (C. Ingram and R. Hessler, personal communication) on filter 1223. These large zooplankton were removed before analysis, as their lipids would dominate those present in the particulate material, masking any potential information in the particulate matter lipid fingerprint. Microscopic zooplankton and parts of zooplankton may still have been present on the filter when analysed, however, and may have contributed to the organic material on the filters. The filter with the highest amount of lipid had no large zooplankton or microscopic organisms when viewed with a $\times 50$ magnification dissecting microscope. This filter had a relatively small amount of water pumped through it apparently because it became plugged due to a large amount of inorganic particulate material.

Five individual compound classes are listed in Table 2 for the five filters analysed for lipids. In general, the lipid fraction of vent samples was dominated by sterols, triacylglycerols, and wax esters (>90% average). The concentrations of all lipid classes increased as the temperature of the water sampled increased. The ratio of an individual compound class to POC was lower in the non-vent water than in the warm vent water (see dive 1228, Table 2), as was noted previously for the ratios of total lipids to POC.

The aliphatic hydrocarbons make up, on average, <3% of the total lipid classes analysed. The major components typically centred around C_{19} or C_{20} (Table 2). The *n*-alkanes dominate this compound class without any marked odd/even carbon predominance. A large unresolved complex mixture indicative of weathered organic material was present in every sample¹⁵. There

Table 2 Particulate organic compound classes

Dive no.	1228	1221	1214	1223	1220
Aliphatic hydrocarbons					
C ₁₈ -C ₃₈ (ng l ⁻¹)	1.40	3.32	5.09	1.14	89.5
CPI*	1.1	1.1	1.0	1.1	1.0
Total/POC(×10 ³)	15.4	23.9	23.3	7.02	408.4
Major components†	25, 21, 22	19, 20, 21	19, 20, 29	19, 20, 18	19, 25, 20
Fatty alcohols					
C ₁₂ -C ₃₀ (ng l ⁻¹)	3.04	12.1	38.5	7.7	57.6
Total/POC(×10 ³)	33.4	86.8	176.3	47.4	263.1
Major components†	18, 16	16, 18:1	18:1, 16	18, 16	18:1, 16
Steryl and wax esters					
C ₃₀ -C ₄₄ total (ng l ⁻¹)	3.17	182.9	375.2	12.0	332.7
Total/POC(×10 ³)	34.8	1,242	1,719	73.8	151.9
Major components†	34:1, 32:1	40:1, 34:1	34:1, 32	34:1	34:1, 32
Triacylglycerols					
C ₄₂ -C ₅₆ total (ng l ⁻¹)	5.34	93.5	500.8	33.8	1,263
Total/POC(×10 ³)	58.6	672.0	2,294	208.4	5,767
Major components†	50, 52	50	50, 52	50, 52	50, 52
Sterols					
Total (ng l ⁻¹)	6.77	23.5	70.0	205.3	1,703
Total/POC(×10 ³)	74.3	168.7	320.5	1,264	7,775
Major components‡	1, 3	1, 2	1, 2	1, 3	1, 2

* CPI, carbon preference index. Odd/even for aliphatic hydrocarbons.

† The numbers indicate carbon number in order of decreasing abundance. Where the numbers are in the form $x:y$, x = number of carbons and y = number of double bonds.

‡ 1 is cholest-5-en-3 β -ol, 2 is cholesta-5,24-dien-3 β -ol, and 3 is 24-methylcholesta-5,24(28)-dien-3 β -ol.

appears to be no clear distinction between the n -alkane distribution in the non-vent and vent water samples that would allow a distinction to be made about the source of the alkanes. The large amounts of aromatic hydrocarbons found in sediments at Guaymas Basin (23–32% of lipid extract)¹⁶ were not present in the particulate material from warm vent waters at 21 °N. This observation, and the large amounts of what appear to be locally biosynthesized lipid compounds (see below), indicate little rapid thermal alteration of organic matter on the suspended particulate material at this vent site. We do not mean to imply that thermal alteration of organic matter does not occur at the 21 °N hydrothermal vents. However, the contribution of thermally altered organic compounds (in the form of aliphatic and polycyclic aromatic hydrocarbons) to the suspended particulate material is in fact very small. Markers for microbial activity in this compound class, such as hopanes¹⁷, isoprenoid hydrocarbons¹⁸, or sterenes¹⁹, were also not present within our limits of detectability.

Free fatty alcohols also make a relatively small contribution to the total lipid (5% average), and were dominated by typical marine biomarker C₁₆, C₁₈, and C_{18:1} alcohols (>74% average)²⁰. The minor components were typically C₁₄, C_{16:1}, C_{20:1} and C₂₄ and on average comprised 12% of the total fatty alcohols. The free fatty alcohols exhibited a high even-to-odd carbon preference (>50).

Both the wax ester and triacylglycerol compound classes make up a large portion of the lipid fraction of organic material (average 56%). The major contribution to the wax ester fraction, C_{34:1} and C₃₂, are typical marine zooplankton wax esters²¹. The high level of these compounds is not surprising considering the large number of macroscopic zooplankton collected from the warm vent water samples. These high values may reflect zooplankton input to the POM or may simply be parts of macroscopic zooplankton or microscopic zooplankton present on the filters that were not picked off. We are unable to distinguish between these two possibilities with our methodology. The concentration of wax esters in open ocean particulate matter, determined with the Woods Hole *In Situ* Pump (WHISP), is 14 ng l⁻¹ (S. G. Wakeham, personal communication) for surface waters from 18° N, 108° W, a moderately productive environment. By comparison, the average of the three vent water values was 240 ng l⁻¹; the non-vent water

sample was ~3 ng l⁻¹. Clearly, the vent particulate matter samples contain over an order of magnitude higher concentration of wax esters than moderately productive surface waters. The warm vent water samples contained both wax and steryl esters, whereas the non-vent and intermediate water filters 1228 and 1221 contained only wax esters, suggesting a warm water source for the steryl esters. This source may be a different species of organism contributing to POM sampled in the warm vent waters.

The steroid alcohols also comprised a major fraction of the total lipid (average 35%). The vent waters were dominated by three sterols (96% total sterols), cholest-5-en-3 β -ol (cholesterol), cholesta-5,24-dien-3 β -ol (desmosterol), and 24-methylcholesta-5,24(28)-dien-3 β -ol (24-methylencholesterol). Non-vent water samples contained mainly cholesterol, typical of other oceanic bottom waters²². The simple distribution of sterols in the vent water samples is not typical of seawater samples previously studied^{9,22}. Pure cultures or single species of organisms usually biosynthesize several major sterols²³. The simple pattern found for the vent samples most probably indicates a select number of single sterol sources. The higher concentration of sterols in warm water relative to non-vent water samples may indicate either an organism source solely living in the warm water or a gradation of productivity between vent and non-vent water. The distribution of the vent water sterols is very similar to that found for molluscs²⁴, suggesting that the clam *Calyptogena magnifica* may be the source. The sterols of *Calyptogena magnifica* are cholesterol, cholestanol and three minor sterols (not identified). This distribution clearly does not match that of the POM, but the sterol composition of the vestimentiferan worm matches extremely well. The major three sterols of the vestimentiferan worm are cholesterol, desmosterol, and 24-methylencholesterol. 24-methylcholesta-5,22E-dien-3 β -ol and 24-ethylcholesta-5,24(28)Z-dien-3 β -ol, sterols known to have a phytoplankton source, were detected in extremely small amounts relative to other sterols.

The initial results from our studies have uncovered several interesting features. All of the vent water samples that we have analysed showed an enrichment of POM relative to non-vent bottom waters. This organic material was both quantitatively and qualitatively different between the non-vent water and vent water, that is the C:N ratio decreased from non-vent to vent

water indicating that the organic material in the non-vent water is more resistant, and represents the less-labile, carbon-rich fraction of the vent POM. The ratio of total lipid/POC also confirmed this conclusion. The lipid class compounds, one of the more labile forms of organic matter, make a larger contribution to the vent water organic carbon than to the non-vent water organic carbon.

The change in concentration of particulate organic matter at this site appears to be a very localized event. In this investigation all samples, including the non-vent sample of bottom water, were taken within an area that was actively emitting warm water and sustaining a large population of benthic organisms. The POC and PON levels measured within the warm waters and in the non-vent water at the vent site vary, in general, by at least a factor of 2–5, the higher values being in warm water. Observations from *Alvin* indicate that the vent organism at 21 °N live in or extremely near to a source of warm vent water, a feature common to other vent sites as well. Our analyses reflect this phenomenon and indicate that primary sources for organic carbon are localized in or near the warm vent water site²⁵.

The relative contribution to the POM by microbial versus animal carbon sources may be partially assessed by using our data on the organic compound source biomarkers. The wax esters, triacylglycerols, and sterols dominate the lipid fraction. The wax ester, triacylglycerol, and fatty alcohol compound classes that were present in the suspended particulate material are typical of marine zooplankton. Indeed, many amphipods and copepods were found in vent waters and collected in our *in situ* pump filters. The sterol distribution on the particulate material matches that of the *Riftia*. The microbial component of the lipid fraction cannot be completely resolved with our data set. Microbially produced lipids, such as hopanols and isoprenoid hydrocarbons, were not found on the filters. Fatty acids, and the more polar phospho- and glycolipids are in the process of being analysed, and these compound classes may contain a large fraction of microbial source markers. Another interesting feature of these analyses is that the typical markers for microbial transformation of sterols such as sterenes, stanones, or stanols, were not present within the limits of our detectability. Thus, we would conclude on the basis of this data set that the zooplankton and larger benthic organisms appear to be making the major contribution to the suspended particulate organic matter in the warm vent waters at 21 °N.

Further verification of both microbial and larger benthic organism contributions and analyses of other source markers will be needed to confirm these initial conclusions from the filtered vent water. Analyses of sediments, bacterial isolates²⁶, and larger organisms from 21 °N are underway to ascertain more specifically the quantitative contributions of sources to the vent water organic matter.

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Experimental manipulation of a contact guidance system in amphibian gastrulation by mechanical tension

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Contact guidance^{1,2} has been implied in various morphogenetic movements including neural crest cell migration³, primordial germ cell migration⁴ and guidance of axonal growth cone⁵. In urodele gastrulae, we reported⁶ the presence of an aligned network of extracellular fibrils on the inside of the ectodermal layer and suggested that it guides the migration of the presumptive mesodermal cells from the blastopore towards the animal pole. We also reported⁷ *in vitro* experiments in which the fibril network of the ectodermal layer was transferred onto the surface of a coverslip. Dissociated mesodermal cells attach to and locomote actively on such conditioned surfaces in an oriented fashion along the blastopore–animal pole axis (bp–ap axis) of the ectodermal layer that conditioned the surface. Recent reports^{8,18} suggest that these fibrils contain fibronectin. We now report that the fibril network on the conditioned surface can be artificially aligned in any orientation by exerting mechanical tension on the ectodermal layer during the conditioning. Such prepared surfaces cause cell movements aligned along the tension axis, even when the tension axis is perpendicular to the natural axis of alignment along the bp–ap axis. These results suggest that the extracellular matrix fibrils aligned by the mechanical stress that arises in embryos during development can orient cell migration by the contact guidance, in a similar manner to that reported in the collagen gel and fibroblasts system^{9,10}.

We used *Ambystoma maculatum* eggs collected in North Carolina and Virginia. Developmental stages were determined according to Harrison stages¹¹. Stage 10– represents very early gastrulae in which the mesodermal cells have not yet started their migration away from the blastopore. In more advanced early gastrulae (stage 10), cell migration is under way. Our previous study⁷ showed that conditioning by stage 10 ectodermal layer causes substantial alignment of mesodermal cell movement, while the conditioning by a stage 10– ectodermal layer

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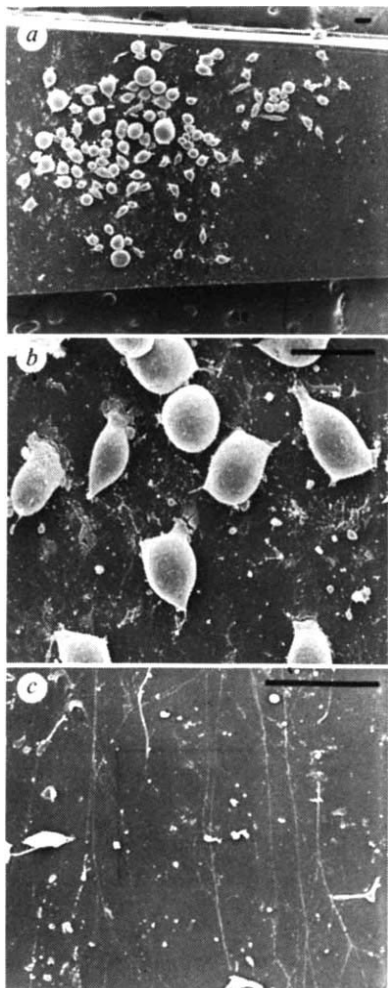


Fig. 1 Scanning electron micrographs of mesodermal cells attached to the conditioned coverslip strip. *a*, A low magnification view. Scale bar, 0.1 mm. *b*, Central part of *a* at higher magnification. Scale bar, 100 μ m. *c*, Extracellular fibrils deposited on the coverslip surface. They are apparently aligned along the axis of tension (vertical axis). Scale bar, 10 μ m. Strips were cut out from plastic coverslips for tissue culture (Thermanox coverslips, Miles) with a sharp steel blade. The composition of media used in this study is the same as used in the previous conditioning experiments⁷. The basic salt solution is a modified Stearns' solution (MSS)^{15,16} buffered with 5 mM HEPES (Sigma). Mesodermal cells were dissociated in 0.02 M sodium citrate¹⁷ in MSS lacking Ca^{2+} and Mg^{2+} . For conditioning of substrata with explants of the ectodermal layer, we used MSS with a pH of 8.0, a Ca^{2+} concentration of 110 μ M, and containing 0.5% bovine serum albumin (BSA) (Sigma). This Ca^{2+} concentration is just high enough to prevent tissue dissociation and yet low enough to prevent curling of the ectodermal fragments, thus it allows the ectodermal fragments to adhere to the substratum and remain flat. The dissociated mesodermal cells were cultured in MSS pH 8.0, a Ca^{2+} concentration of 100 μ M, and containing 0.5% BSA, which we have found to be optimal for cell locomotion.

gives only very weak alignment. This difference may be caused by the alignment of the fibril network by the expansion and stretching of the ectodermal layer along the bp-ap axis during gastrulation¹².

Rectangular pieces of the ectodermal cell layer were cut out from the whole blastocoelic roof of early gastrulae (stage 10–, or 10) using fine forceps and hair loops. The pieces were explanted on the coverslip strips (2 mm $\times$ 10 mm), which were set on top of two small fragments of glass capillary (outer diameter, 2 mm) like a bridge, so that the explant could be hung with both ends drooping free from the edges of the strip, thus producing constant mechanical tension perpendicular to the longer axis of the strip of coverslip. The inner surface of the ectodermal

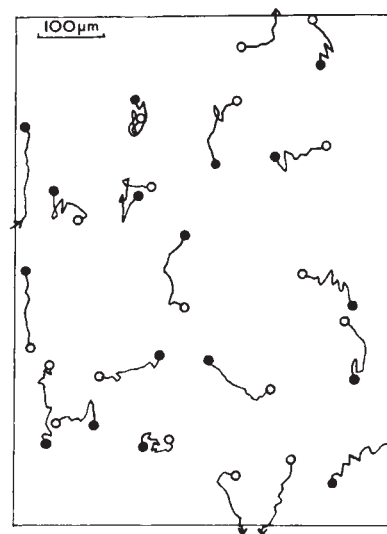


Fig. 2 Cell trails drawn from a time-lapse film of the tilted conditioning. O, Starting points; ●, finishing points after 1 h. They are apparently aligned along the axis of tension (vertical axis). The overall *R* value calculated for all the cell trails here is +0.47. Time-lapse 16-mm films were taken with $\times 10$ phase contrast lens at 8-s intervals. At the end of filming, the coverslip was fixed and processed for scanning electron microscopy⁷. We drew cell trails by projecting the time-lapse films and marking the centre of cell body at 4-min intervals¹⁵.

layer adhered to the coverslip surface. The strip of coverslip had been set horizontally or tilted by approximately 30° by applying a small amount of Vaseline between the glass capillary and the strip. The tilting allowed the mechanical tension to be maintained throughout the explant during the conditioning. In the horizontal setting, both edges of the coverslip held the ectodermal layer tightly and prevented the tension from transmitting to the central part of the explant. On the other hand, with tilting, there was one free edge so that the tension and stretching generated by the weight of the explant could be transmitted across the entire sheet. Explants from stage 10 embryos were placed on the coverslip with their bp-ap axis parallel to the longer axis of the strip. Explants from stage 10– embryos were oriented randomly, because they have not yet developed their natural alignment along the bp-ap axis⁷.

After 5 h of culture at room temperature (22–24 °C), explants were removed from the coverslip by flushing them away with a Pasteur pipette. The coverslip strips were rinsed with culture medium, and placed horizontally in a plastic Petri dish. They were seeded with dissociated mesodermal cells from middle gastrulae (stage 10+) using a Pasteur pipette. The cells attach to the conditioned surface and start to migrate actively within 30 min. The mesodermal cells show no attachment outside the conditioned area. Figure 1*a* shows a low magnification view of a coverslip strip and attached cells. The cells frequently have a leading end with a large lamellipodium and associated filopodia and a slender or rounded trailing end (Fig. 1*b*). The conditioned surface is covered with fragments of cell-surface structures such as filopodia and a network of extracellular fibrils that can be observed only at higher magnifications (Fig. 1*c*). The network was apparently aligned along the axis of tension (Fig. 1*c*). In fact, when scanning electron micrographs of 10 randomly selected areas from the tilted conditioning were analysed by the microcomputer system (see below), they gave positive *R* values; mean and standard deviation were $+0.74 \pm 0.52$.

Cell trails from the tilted conditioning showed an apparent alignment along the axis of tension (Fig. 2). No alignment was noticeable when we used the horizontal conditioning. We further analysed the cell trails by using an image-analysing system with a microcomputer (Apple II Plus Computer and Graphics Tablet)⁷. The computer programs⁷ we used enable us to determine

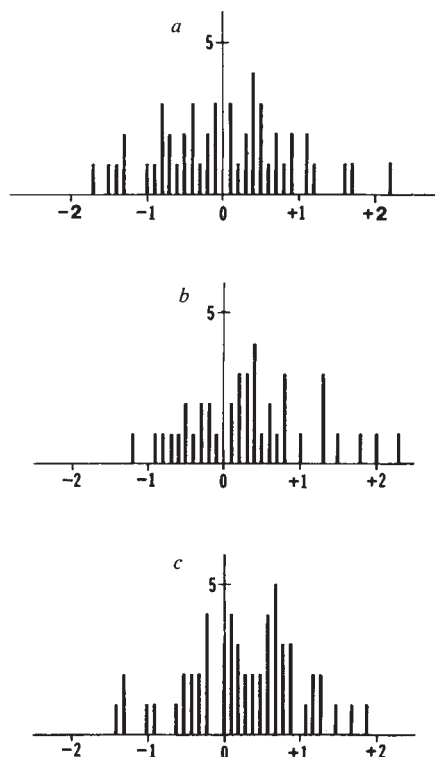


Fig. 3 Histograms showing distribution of cell trails according to their R values. Horizontal axis, R value; vertical axis, number of cell trails. *a*, Data from the horizontal conditioning, showing nearly normal distribution centred at zero (mean -0.01 and s.d. ± 0.90 , 52 cells). *b*, Tilted conditioning using the stage 10–ectodermal layer. The distribution is clearly shifted to the right (mean $+0.36$, s.d. ± 0.78 , 40 cells). The shift of mean is significant ($P < 0.005$). *c*, Tilted conditioning using the stage 10 ectodermal layer. The shift to the right (mean $+0.29$, s.d. ± 0.73 , 55 cells) is significant ($P < 0.005$).

whether the cell trails are aligned along the axis of the mechanical tension during the conditioning. Also, they were used to analyse alignment of the fibril network deposited on the conditioned surface. The programs calculate displacement (Δy and Δx) along the y -axis (axis of tension) and along the perpendicular x -axis in each short segment of the cell trail, and give sums of $|\Delta y|$ and $|\Delta x|$ separately for the whole cell trail. Then, it gives R values, $R = \log_2(\Sigma|\Delta y|/\Sigma|\Delta x|)$, as a parameter of the alignment; a zero value for non-aligned random movement, positive values of R for movement aligned along the axis of tension, and negative values for movement aligned perpendicular to the axis of tension. Figure 3 shows histograms of the distribution of cell trails according to their R values. When horizontal conditioning was used (Fig. 3*a*) we observed nearly normal distribution centred at zero, showing that the cell trails are not aligned. On the other hand, when we used tilted conditioning (Fig. 3*b*, *c*) we observed a distribution that was shifted to the right (positive values). The shift of the mean ($+0.36$ and $+0.29$) is larger than that of the natural alignment along the bp–ap axis in the stage 10 ectoderm conditioning ($+0.22$)⁷. Substratum conditioning using stage 10–ectodermal layers (Fig. 3*b*) gives stronger alignment than that using stage 10 ectoderm (Fig. 3*c*). The most likely explanation for this is that the stage 10 explant was oriented on the coverslip strip in such a way that its natural alignment along the bp–ap axis must be cancelled before any appearance of the alignment along the axis of tension.

Contact inhibition of cell movement is another mechanism that can direct cell migration from the blastopore with a high population of migratory cells towards the animal pole where the fibril network on the inner surface of the ectodermal layer offers an adequate substratum for cell attachment and locomotion.

This possibility has been suggested^{6,7,13,14}, and the gastrula mesodermal cells indeed show contact paralysis against each other when cultured *in vitro*^{13,15}.

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Forskolin activation of adenylate cyclase *in vivo* stimulates nerve regeneration

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The previous demonstration of an increase and redistribution of adenylate cyclase activity in injured peripheral nerve¹ suggests that an increase in neuronal cyclic AMP concentration could play a role in peripheral nerve regeneration. We report our finding that accumulating adenylate cyclase activity was translated into a twofold increase in cyclic AMP concentration in the regenerating nerve stump, coincident with the initiation and elongation of regenerative nerve sprouts. We sought to magnify the role of cyclic AMP in regeneration by using forskolin, a robust activator of adenylate cyclase², to produce an additional increase in neuronal cyclic AMP *in situ*. Forskolin *in vitro* produced an approximately 40-fold greater elevation in neuronal cyclic AMP than an equimolar (10^{-5}) concentration of isoprenaline. Moreover, the elevated cyclic AMP concentration persisted for at least 60 min in the continued presence of forskolin. Daily injection of forskolin into the dorsal lymph sac of *Rana pipiens*, or delivery of forskolin through an implanted osmotic pump produced a sustained 40% increase in the rate of sensory nerve regeneration in freeze-lesioned sciatic nerves. We conclude that an increase in cyclic AMP concentration and, presumably, the activation of appropriate protein kinases stimulates regenerative nerve growth following trauma.

Adenylate cyclase, the enzyme responsible for catalysing the formation of cyclic AMP from ATP, undergoes anterograde, but not retrograde axonal transport in normal frog sciatic nerve. If the nerve is subjected to crush or transection, however, adenylate cyclase activity accumulates proximal to the site of injury and is incorporated in the retrograde transport system for return toward the cell body¹. While these observations provide the basis for a stimulated production of cyclic AMP in regenerating nerve, increased production does not necessarily lead to increased concentration. A parallel elevation of phosphodiesterase activity, an enzyme which also undergoes axonal transport³, could prevent accumulation of the cyclic nucleotide. We measured cyclic AMP concentration in specific regions of axotomized frog sciatic nerve at 2, 4 and 7 days after a freeze-

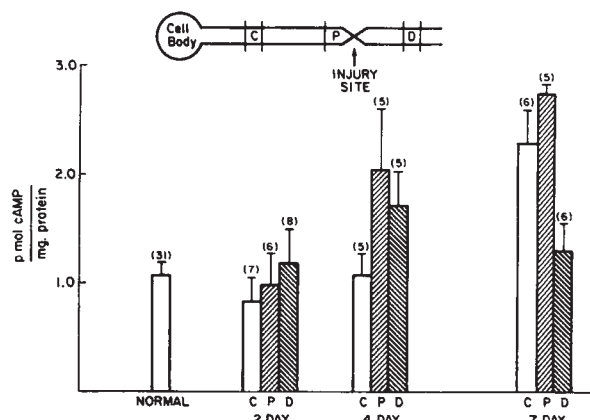


Fig. 1 The concentration of cyclic AMP in 3–4 mm desheathed nerve segments removed from indicated regions of normal and freeze-lesioned sciatic nerves at specified times following the injury. Numbers in parentheses represent the sample size for each measurement. Sample P was obtained just proximal to the injury and includes regenerative growth at 4 and 7 days. Samples C and D were removed from regions 1 cm proximal and 1 cm distal to the injury respectively.

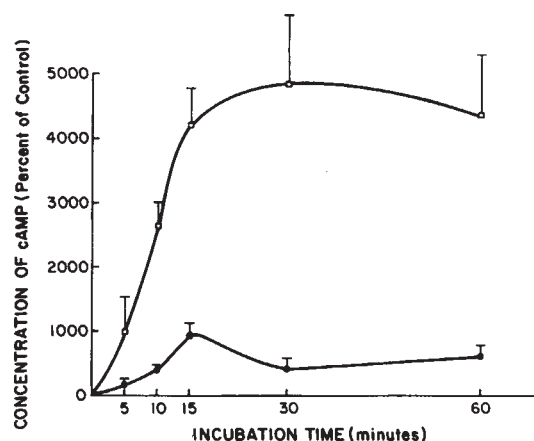


Fig. 2 Comparison of the relative stimulatory capabilities of equimolar concentrations (10^{-5} M) of forskolin (Calbiochem) ($\square$) and isoprenaline ($\bullet$) applied to desheathed 3–4 mm segments of normal sciatic nerve *in vitro*. Each time point represents the mean $\pm$ s.e.m. of four measurements. Nerve segments were preincubated for 1 h at room temperature in normal frog Ringer's solution through which a mixture 95% O_2 :5% CO_2 was continuously bubbled. The preincubation solution was removed and fresh solution added for a second 15 min preincubation. The drug was then added for the specified period of time, at the end of which the nerve segment was frozen and cyclic AMP extracted for radioimmunoassay. Each stimulated segment was paired with an unstimulated control.

lesion of the nerve. These post-injury intervals were chosen to reflect the period prior to regenerative sprouting (2 days), the period of sprout initiation (4 days) and the early period of axon elongation (7 days). The results of these experiments are presented in Fig. 1. Freeze-lesioned or intact sciatic nerves were removed from northern *Rana pipiens* (30–50 g body weight, mixed sex, obtained from J. Hazen, Alburg, Vermont, USA, during Winter and Spring). The isolated nerve was placed in Ringer's solution and desheathed. Desheathed nerve segments, 3–4 mm in length, were obtained from the injury site (including regenerative sprouts) and from regions 1 cm proximal and 1 cm distal to the injury. Each segment was incubated in a separate flask containing Ringer's solution without a phosphodiesterase inhibitor for 1 h at room temperature (21–22 °C). The solution was continuously supplied with a mixture of 95% O_2 and 5% CO_2 . At the end of the incubation period the segment was rapidly frozen in a mixture of ethanol:hydrochloric acid (60:1, wt/wt) cooled on dry ice (–70 °C). The frozen tissue was homogenized and prepared for analysis of cyclic AMP concentration by radioimmunoassay⁴. Protein concentration was determined using the Lowry assay. The mean concentration of cyclic AMP in segments from the selected locations were compared statistically to segments from similar regions of intact nerves using analysis of variance and Dunnett's *t*-test.

Cyclic AMP concentration was not significantly different from normal nerve in any of the three sample regions of axotomized nerve 2 days after injury. Thus, the early accumulation of adenylate cyclase activity at the injury site¹ was not translated into an equivalent increase in cyclic AMP concentration prior to the development of regenerative sprouting. After 4 days, however, cyclic AMP concentration was significantly ($P < 0.05$) increased in the region of regenerative growth, when regenerative sprouts are first appearing³. Concentration also increased in segments distal to the injury at 4 days, although the increase was not statistically significant. At 7 days, cyclic AMP concentration was significantly increased in nerve segments encompassing regenerative growth, and in segments 1 cm proximal to the injury ($P < 0.01$). Concentration in segments distal to the injury had returned to control values.

This pattern of change in cyclic AMP concentration supports our hypothesis that an increase in cyclic AMP concentration may play an integral role in nerve regeneration. Previous studies, however, in which dibutyryl cyclic AMP was injected into rats during the course of peripheral nerve regeneration offer mixed results^{6–9}. The treatment-enhanced regeneration in two cases^{6,7}, but had no effect in two others^{8,9}. None of these studies assessed

the actual cyclic AMP concentration in the regenerating nerve, or the magnitude of the presumed increase resulting from the injection of dibutyryl cyclic AMP. We chose to explore the possible role of cyclic AMP in regeneration by using an extremely potent activator of adenylate cyclase, forskolin (Calbiochem), to increase endogenous levels of cyclic AMP in the injured nerve. Forskolin stimulates adenylate cyclase via the catalytic subunit of the enzyme², and produced an increase in activity two to three times that produced by sodium fluoride *in frog nerve* (unpublished observation). Incubation of desheathed nerve segments from normal frog nerve in media containing either 10^{-5} M forskolin or 10^{-5} M isoprenaline demonstrated that forskolin could produce an increase in cyclic AMP concentration 40-fold greater than that produced by isoprenaline (Fig. 2). Moreover, the increase in concentration was sustained for at least 60 min in the continued presence of forskolin.

The effect of forskolin on nerve regeneration was tested in frogs subjected to a unilateral freeze-lesion of the sciatic nerve at mid-thigh. Forskolin was provided either by daily injection of 0.5 ml of a 5×10^{-4} M solution into the dorsal lymph sac, or by implanting an osmotic pump (Alza) which delivered 0.55 μ l per h (21 °C) of a 10^{-4} M solution in the vicinity of the injury. Animals with an implanted pump also received a single 'priming' injection of the 5×10^{-4} M forskolin solution (0.5 ml). Control animals received daily saline injections or had implanted osmotic pumps containing a 0.64% saline solution. Regeneration distance in both forskolin-treated and control animals was determined using axonal transport of H^3 -leucine-labelled protein from lumbar dorsal root ganglia to determine the maximum extent of sensory nerve outgrowth^{5,10}. Ganglia and attached spinal and sciatic nerves were removed and placed in a compartmented chamber¹¹. The ganglia were contained in a centre compartment and the nerves passed through a silicone grease barrier into an outer compartment. The tissue was bathed in aerated Ringer's solution and 50 μ Ci ml^{-1} of 3H -leucine added to the ganglia compartment. Following a 3–4 h incubation period the isotope was removed, the tissue washed and placed in a second chamber; axonal transport was then allowed to continue for an additional 16–18 h. At the end of the transport period the nerve was frozen and divided into 1- or 2-mm segments. Each segment was placed in 5% trichloroacetic acid (TCA)

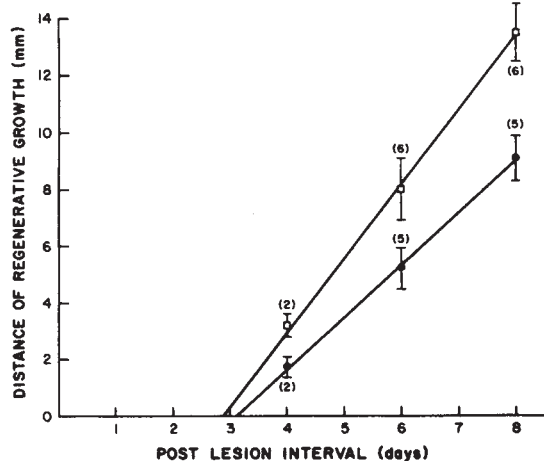


Fig. 3 Measurement of regeneration distance in axotomized sensory nerves from animals receiving forskolin and from saline-treated controls. Regeneration distance was determined at the indicated time points by assaying the distribution of axonally-transported radioactively labelled protein in the regenerating nerves. Distances at each time point represent the mean $\pm$ s.d. of the indicated number of measurements. Distance points were connected by linear regression lines using the method of least squares. The slope of each line indicates the regeneration rate in forskolin-treated and control, saline-treated sensory nerves. For treated nerves the slope is 2.6 mm per day, and for controls, 1.86 mm per day.

overnight to separate TCA-soluble and insoluble material. The soluble material was discarded and the amount of TCA-insoluble radioactivity determined by liquid scintillation spectroscopy. Regeneration distance was taken as the point at which sample counts declined to within two standard deviations of mean background activity¹⁰. Forskolin-treated frogs showed a 40% increase in the rate of sensory nerve regeneration without a statistically significant change in latency to the initiation of regenerative growth. Enhancement of regenerative growth was the same using either of the two modes of drug application (results are pooled in Fig. 3). Regeneration rate in forskolin-treated nerves was significantly greater ($P < 0.001$) than the rate in saline-treated animals, as determined by linear regression analysis¹². Forskolin-stimulated enhancement of regeneration also exceeded the 12–17 percent enhancement obtained using the condition/test paradigm⁹ in frogs at 25 °C¹².

Functional recovery following nerve injury requires both axonal regrowth and reconnection with appropriate target organs. The processes responsible for recovery are complex and require interaction between the injured axon, its cell body, periaxonal cells in the axon and, eventually, cells in the target organ¹³. In the present experiments we attempted to link an increase in cyclic AMP concentration in regenerating axons to the development of regenerative growth. We determined that forskolin, an agent which stimulates cyclic AMP production also enhances the rate of axon regeneration. The site of action of forskolin is currently unknown, but could involve processes in either or both the axonal injury site or the nerve cell body. Forskolin does not cross the blood-brain barrier², but dorsal root ganglia neurones are not contained within the barrier¹⁴ and injected forskolin should be able to interact with injured sensory cell bodies. The role of cyclic AMP in regeneration is also unknown, although an increase in cyclic AMP might stimulate microtubule aggregation to enhance sprout formation¹⁵ or provide a modulating influence on RNA and protein synthesis in the cell body¹⁶. While the rate of nerve regeneration is but one aspect of the recovery process, stimulation of rate, particularly in human nerve injuries located far proximal in a limb, might improve recovery by allowing re-innervation of target tissue before target organ degeneration has become extensive¹⁷. We are currently investigating the specific molecular role of

cyclic AMP in injured nerve, but it appears clear that the nucleotide can play an important role in axonal growth.

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Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization

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Botulinum neurotoxin (BoNT) type A, a causative agent of botulism, is a di-chain protein (molecular weight 140,000) from *Clostridium botulinum*, and the most neurotoxic substance known¹. Some cases of sudden infant cot deaths have been attributed to such a neuromuscular condition². BoNT inhibits irreversibly the release of acetylcholine from peripheral nerves in a highly selective manner^{3–6}. Hence, it is potentially an invaluable probe for studying the mechanism of transmitter release^{7,8}. Here we demonstrate specific labelling of murine motor nerve terminals with neurotoxic, ¹²⁵I-labelled BoNT (type A) by autoradiography. We observed saturable, temperature-sensitive binding of BoNT to sites which reside solely on the nerve terminal membrane; these were distributed on all unmyelinated areas, at an average density of 150–500 per μm^2 of membrane. The binding was mediated by the larger subunit of the toxin and was inhibited partially by tetanus toxin, another microbial protein⁹. No specific binding was detectable on any other cell types examined, including noradrenergic terminals. Following binding, internalization of radioactivity was observed; this process was energy-dependent as it could be prevented totally by azide or dinitrophenol (DNP). This direct demonstration of separable steps, including highly selective binding and acceptor-mediated internalization, is reconcilable with the unique potency and the multiphasic inhibitory action of BoNT on transmitter release, as shown electrophysiologically⁸.

To facilitate characterization of BoNT-binding sites on neuronal membranes, with the long-term goal of elucidating the molecular basis of its action, BoNT type A was purified to homogeneity and ¹²⁵I-iodinated to high specific radioactivity (700–3,000 Ci mmol⁻¹). The resultant BoNT (¹²⁵I-BoNT) retained appreciable levels of neurotoxicity (60–85% of the original, equivalent to 2×10^8 mouse lethal doses per mg protein)^{7,10} and was shown to bind saturably and with high affinity to a sialic acid-containing moiety on brain synaptic membranes¹⁰. Additional evidence of its biological activity was provided by the demonstration, using light microscope autoradiography, that it binds at low concentrations to a discrete

Fig. 1 Electron microscope autoradiograms of phrenic nerve muscle preparations labelled with ^{125}I -BoNT. *a, c, e*, With tetanus toxin; *b, d, f*, without. *a*, Sample treated with ^{125}I -BoNT alone. *b*, Control sample labelled with ^{125}I -BoNT and excess unlabelled toxin. *c*, Muscle preincubated at 22 °C for 20 min in Krebs-Ringer's containing 15 mM sodium azide and then labelled in the latter medium with ^{125}I -BoNT as in *a*. *d*, A control sample of tissue treated with sodium azide as in *c* then incubated with labelled and unlabelled BoNT as in *b, f*. Specimen labelled with ^{125}I -BoNT as in *a* except that the incubation period with toxin was 2 h. *e*, The preparation was preincubated with 0.9 μM tetanus toxin; ^{125}I -BoNT was then added and the incubation continued for a further 2 h.

Methods: Each dissected mouse hemidiaphragm was incubated with 11 nM ^{125}I -BoNT in 0.5 ml Krebs-Ringer's pH 7.4 at 22 °C for 1.5 h unless otherwise specified; use of a 3 ml incubation volume yielded similar results. Control samples were treated similarly except that 1.1 μM BoNT was added together with the radiolabelled toxin. Labelled tissues were washed repeatedly in Krebs-Ringer's for 30 min at 22 °C before fixation for 1 h with 2% glutaraldehyde in the latter solution. Endplate-containing regions of each sample were cut into 1-mm³ pieces, treated with 1% OsO₄ for 1 h, dehydrated in ethanol and embedded in Spurr's resin. Pale gold or silver sections were cut using an LKB ultramicrotome and processed for autoradiography¹³; a monolayer of L4 emulsion was applied to the sample by dipping. Sections were exposed for 3 weeks, developed in Kodak D-19 developer, stained with 12% uranyl acetate, examined in a Hitachi electron microscope and photographed.

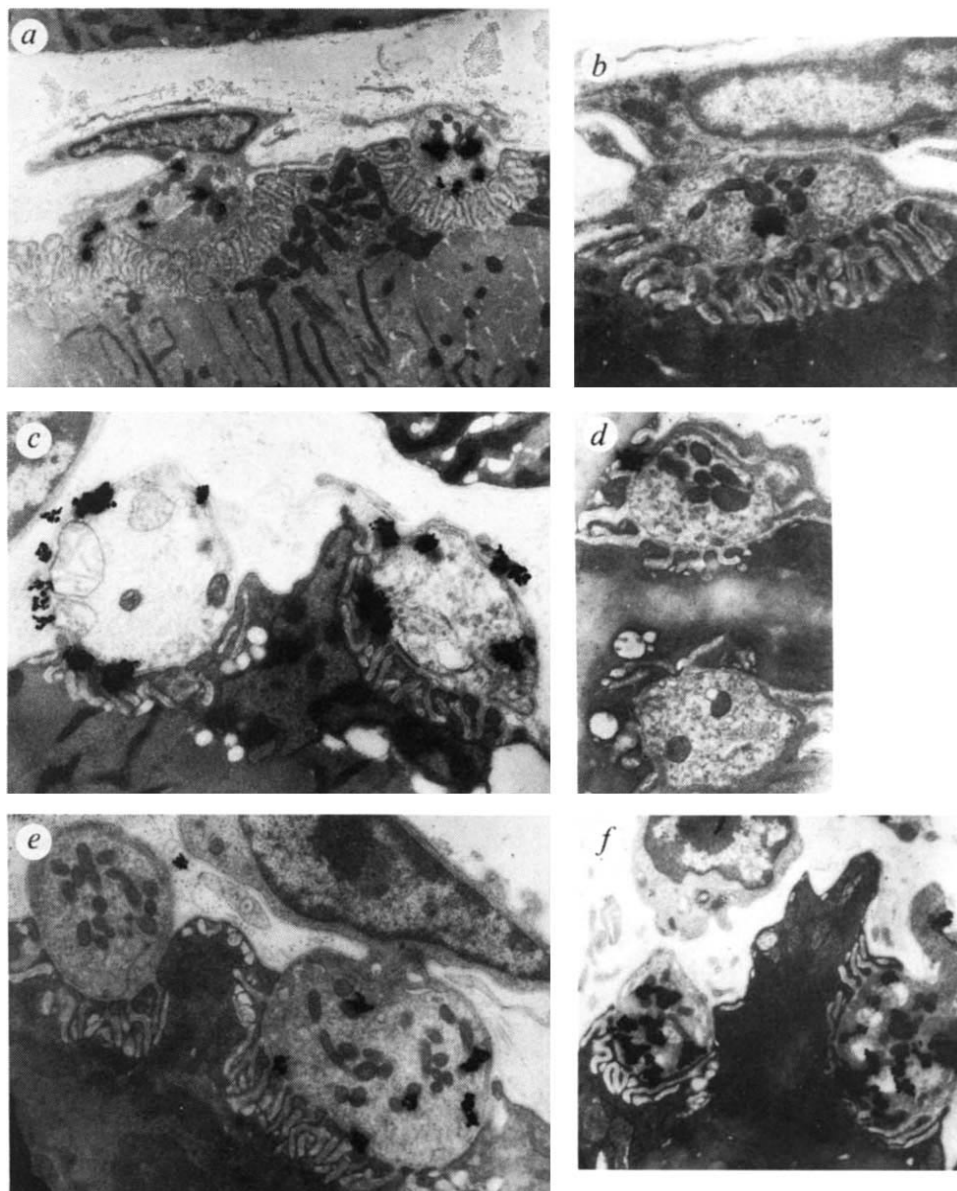


Table 1 Autoradiographic analysis of nerve terminals labelled with ^{125}I -BoNT

Incubations	Distribution of grains with respect to the plasma membrane (% of total)		Relative number of grains/unit area of plasma membrane (%)
	On*	Within	
Series I (28 nM ^{125}I -BoNT)			
No additives	62 (± 16 ; $n = 15$)	38 (± 16 ; $n = 15$)	100†
1.4 μM BoNT	—	—	4
-Ca ²⁺ /+2 mM EGTA	61 (± 24 ; $n = 21$)	39 (± 24 ; $n = 21$)	74
Series II (11 nM ^{125}I -BoNT)			
No additives	61 (± 17 ; $n = 22$)	39 (± 17 ; $n = 22$)	100†
1.1 μM BoNT	—	—	5
0.5 μM BoNT large subunit	—	—	0
15 mM sodium azide	100 ($n = 50$)	—	85
0.9 μM tetanus toxin	61 (± 25 ; $n = 23$)	39 (± 25 ; $n = 23$)	31
Series III (1 nM ^{125}I -BoNT)			
No additives	54 (± 24 ; $n = 6$)	46 (± 24 ; $n = 6$)	100†
50 nM BoNT	—	—	0

Mouse hemidiaphragms were labelled with ^{125}I -BoNT for 1.5 h at 22 °C in 0.5 ml of Krebs-Ringer's solution in the conditions specified. Tissues were subsequently washed, fixed, embedded and pale gold sections were cut; autoradiograms of these were prepared and developed after 3 weeks' exposure, as detailed in Fig. 1. After examination in a Hitachi electron microscope, large numbers of nerve endings in several sections were photographed; the silver grains in each were counted and the mean values ($\pm$ s.d., n = number of terminals counted) are expressed. The samples in each series of experiments were treated identically.

* 90% of these grains were found on the membrane itself; 10% were within 150 nm of the latter and can be attributed to expected scatter.

† The area of the terminal membrane in each section was calculated by digitization and the total number of grains observed on the membrane and enclosed within it were expressed per unit area of the membrane; these were taken to represent 100% in each case.

number of sites at murine motor endplates^{7,11}, following *in vivo* or *in vitro* labelling; a similar observation has been made for another preparation of ¹²⁵I-BoNT¹².

To locate the site of this binding, mouse hemidiaphragms were incubated with ¹²⁵I-BoNT, washed and processed for autoradiography¹³ before examination by electron microscopy. To measure nonspecific binding of ¹²⁵I-BoNT, samples were treated similarly except that an excess of BoNT was included. After labelling of nerve-muscle preparations with ¹²⁵I-BoNT *in vitro*, in conditions which block neurotransmission⁵, autoradiography showed a distinct deposition of silver grains at the motor nerve endings (Fig. 1a). This labelling was saturable as control samples treated with excess BoNT contained few, if any, silver grains (Fig. 1b; Table 1). All the sites were occupied when 50 nM ¹²⁵I-BoNT was used, in labelling conditions (1.5 h; 22 °C) that minimize structural damage to the tissue. Greatly reduced numbers of grains were seen when the incubation was done at 4 °C; we have been unable to ascertain how much changes in rate of diffusion contribute to this.

A similar qualitative deposition of grains was observed when low amounts (0.5 nM) of ¹²⁵I-BoNT were used, although fewer grains were apparent. This does not preclude the possibility that the acceptors have a high affinity for BoNT, as even higher concentrations (~1 µM) of α-bungarotoxin ($K_D \sim 10^{-11}$ M) are similarly required to overcome diffusion barriers in this tissue¹⁴, within a similar incubation period. In all cases, the labelling with ¹²⁵I-BoNT was associated selectively with nerve terminals. Muscle, blood vessels, connective tissue, Schwann cells and myelin were virtually devoid of grains (Fig. 1); the number of grains observed was insignificant and these were distributed randomly, their presence being unaffected by excess unlabelled BoNT. Furthermore, it proved impossible to demonstrate any deposition of silver grains on noradrenergic terminals (Fig. 2) of mouse vas deferens¹⁵, despite use of high ¹²⁵I-BoNT concentrations.

The saturable labelling of nerve terminals demonstrated with ¹²⁵I-BoNT was restricted to the presynaptic membrane (Fig. 1, Table 1); using the standard incubation conditions, most grains (62 ± 16% of the total) were located on the nerve membrane, the remainder being inside the terminal. Transfer of radioactivity across the plasma membrane was detectable after incubation with 11 nM toxin for 20 min, but the extent of internalization reached a maximum after 90 min; at present, we do not know whether the grains seen represent the intact toxin molecule or one of its subunits. However, when a homogeneous preparation of the non-toxic larger subunit of BoNT was included in the incubation medium, after separation¹⁰ and renaturation (neurotoxic material can be reconstituted by the addition of the smaller subunit [55,000 molecular weight] to this preparation¹⁶), there was no ¹²⁵I-BoNT labelling (Table 1). This showed that the heavier subunit (97,000 molecular weight) is responsible for the observed binding, as is the case for binding to brain synaptosomal membranes¹⁰. The kinetics of the toxin's neuromuscular action are consistent with the involvement of a target site within the terminal¹⁷ and the findings presented here provide the first direct evidence for such internalization of BoNT. Lowering the temperature to 4 °C abolished internalization of radioactivity, as did inhibitors of energy production such as azide (Fig. 1c, Table 1) or DNP. This indicates that the overall process is energy requiring; we do not know which of the many possible mechanisms¹⁸ is involved. In these conditions the labelling was saturable (Fig. 1c and d); although the total number of grains observed was reduced somewhat, they were now all deposited on the neuronal membrane (Table 1) or, at least, very close to it. Tetanus toxin was tested as a possible antagonist for the BoNT acceptor since its structure⁸ is comparable and it has a related pharmacological action^{9,19}. The labelling of nerve terminals was reduced significantly in the presence of tetanus toxin (Fig. 1e and f); however, the fraction of radioactivity internalized was unaffected (Table 1). Thus, the inhibitory action of tetanus toxin, together with that of the large BoNT subunit, on the labelling observed further supports the saturabil-

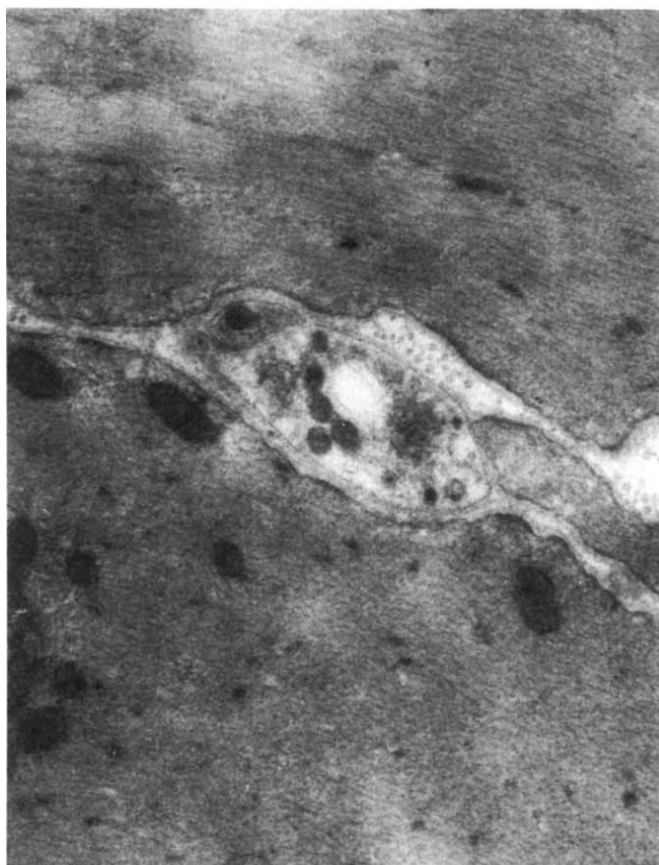


Fig. 2 Autoradiography of noradrenergic terminals from mouse vas deferens treated with ¹²⁵I-BoNT. The serosal coat of the dissected vas deferens was removed by gentle pulling and the remaining tissue incubated with 22 nM ¹²⁵I-BoNT in 0.5 ml Krebs-Ringer's for 90 min at 22 °C. The tissue was washed with Krebs-Ringer's for 30 min, then it was stretched and held in place with pins before being fixed for 90 min at 22 °C with 3% glutaraldehyde/2% paraformaldehyde/0.1 M sodium cacodylate buffer, pH 7.4. The sample was cut transversely into small segments and these were washed twice for 20 min in 5% sucrose/0.1 M sodium cacodylate buffer, pH 7.4 before treatment for 1 h at 22 °C with 2% OsO₄ in the latter buffer. This fixation method yields maximum preservation of dense core vesicles which facilitate identification of the noradrenergic terminals. After washing, the sample was dehydrated, embedded and prepared for autoradiography as described in Fig. 1 legend. The sections were developed after 3 weeks' exposure, viewed in the electron microscope and photographed. Dense core vesicles, which are characteristic of noradrenergic nerve terminals, were observed but no silver grains were seen in any area of the sections.

ity of the BoNT acceptor. Collectively, the findings demonstrate the existence of at least two distinguishable and sequential steps—toxin binding and internalization.

With toxin uptake inhibited, the content of binding sites could be quantified readily; using an established procedure¹³, the average density was calculated to be 150–500 sites per µm² of membrane. Also, with azide present it was apparent that the binding sites are distributed uniformly over all the unmyelinated areas of the terminal membrane (Fig. 1c). Hence, in contrast to previous suggestions²⁰, it appears unlikely that the binding sites are involved directly in the process of transmitter release because their location is not restricted to the areas of membrane (active zones) opposing the postsynaptic folds, where release is thought to occur. It should be emphasized that with a low concentration of ¹²⁵I-BoNT (1 nM), in the presence of azide, the grain distribution obtained was similar; in fact, examination of autoradiograms of 21 endplates labelled in this manner showed that in 16, grains were not restricted to the active zone regions (as was seen also with higher toxin concentrations). Nevertheless, the apparent selective location (Figs 1 and 2) of

the binding site on cholinergic nerve membranes (at least in the periphery) indicates some, as yet unidentified, role. At least it seems clear from the de-energization experiments (Fig. 1c, Table 1) that the binding step is essential for translocation of the toxin into the nerve terminal. Furthermore, in the case of noradrenergic synapses⁶ where BoNT is unable to inhibit neurotransmission at concentrations known to inhibit acetylcholine release at motor nerve terminals²¹, the demonstrable absence of any binding sites for BoNT could render the transport of toxin across the plasma membrane to its site of action impossible. The minimal inhibitory effects on noradrenergic transmission in the anococcygeus muscle, seen after application of BoNT for long periods⁶, may result from its inefficient uptake by another route. Similarly, absence of the toxin acceptor could explain why non-adrenergic, non-cholinergic transmission in guinea pig taenia coli is unaffected by BoNT⁶. It is likely that the intraterminal target may be common to several synapse types. Finally, the ability of tetanus toxin to antagonize BoNT binding emphasizes the importance of the toxin-binding step, as there are many similarities in the actions of these toxins at motor endplates¹⁹.

Although all available pharmacological data⁸ and the findings reported here support the contention that toxin translocation is essential for its neurotoxicity, additional evidence was sought using conditions known to interfere with the neuromuscular action of BoNT, without perturbing its binding. In contrast to deductions from electrophysiological investigations¹⁷, absence of Ca^{2+} from the incubation medium did not appreciably inhibit the internalization (or binding) of BoNT (Table 1). Similarly, lysosomotropic agents (for example, chloroquine) which are effective antagonists of botulism²¹ failed to prevent BoNT translocation (data not shown). Chloroquine is known to retard the expression of the activity of other microbial toxins²², following their internalization by energy-dependent process(es) of the type described here. Thus, it is likely that this ability (which involves increasing the pH of lysosomes²² or possibly of other compartments²³), rather than its proposed effectiveness in inhibiting uptake²¹, is implicated in delaying the onset of neuromuscular paralysis by BoNT. With regard to the individual steps of botulinization, the resolution of autoradiographic techniques cannot exclude membrane sequestration of toxin following its binding to external sites. However, it is not likely that this could allow direct access of toxin to its pharmacological target because of the demonstrated internalization of toxin and its sensitivity to chloroquine. Thus, intoxication seems to depend on toxin uptake; the associated intraterminal events could facilitate a detrimental interaction of BoNT, or a fragment of it, with a component essential for transmitter release. This final target might reside within the active zones; in fact, it could be responsible for the presence of some silver grains in autoradiograms of terminals labelled with ¹²⁵I-BoNT in the absence of azide (Fig. 1a and f).

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L-Glutamate has higher affinity than other amino acids for [³H]-D-AP5 binding sites in rat brain membranes

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Electrophysiological studies indicate the existence of several types of receptors for excitatory amino acids¹. Thus, responses induced by *N*-methyl-D-aspartate (NMDA) are potently and selectively blocked by D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), while responses induced by such agonists as kainate and quisqualate are relatively resistant to this antagonist^{2,3}. Evidence is mounting that excitatory amino acid receptors are involved in synaptic excitation in many regions of the central nervous system (see refs 1 and 4 for reviews). Although the identity of the transmitter(s) acting at these receptors remains uncertain⁵, L-aspartate has been considered the most likely transmitter at NMDA receptors and L-glutamate at kainate/quisqualate receptors^{1,4}. Other endogenous acidic amino acids proposed as possible transmitters include a range of sulphur-containing amino acids⁶ and the tryptophan metabolite, quinolinic acid⁷. Ligand-binding studies offer a means not only of assessing receptor densities in different brain regions but also of comparing affinities of transmitter candidates for these receptors. However, to avoid difficulties of interpretation arising from the use of ligands which bind to more than one type of receptor, such as [³H]-L-glutamate and [³H]-L-aspartate (for example, refs 8–12), ligands with high receptor selectivity are required. Here, we report that [³H]-D-AP5 binds specifically to rat brain membranes, that the hippocampus and cerebral cortex are enriched in these sites relative to other brain areas and that L-glutamate has higher affinity for these receptors than have all other transmitter candidates tested.

[³H]-D-AP5 binding to rat cerebral cortical membranes reached equilibrium following 20 min incubation at 25 °C. Specific binding contributed about 40% to the total binding at 30 nM [³H]-D-AP5. This binding was reversible, the bound ligand being completely displaced by 1 mM unlabelled ligand added at 25 min with a $t_{1/2}$ of 5 min. The kinetics of D-AP5 binding were investigated by incubating membranes with 17 nM [³H]-D-AP5 in the presence of increasing concentrations of unlabelled D-AP5 (0.05–100 μM); computer fitting of the data revealed a single population of receptors (Fig. 1). Analysis of results from four such experiments gave K_D and B_{max} values of 0.47 ± 0.09 μM and 4.1 ± 0.7 pmol per mg protein respectively (mean values $\pm$ s.e.). D-AP5 binding sites were present in all areas of the CNS tested. There was, however, a marked enrichment especially in the hippocampus, but also in the cerebral cortex (Fig. 2). Specific binding was lowest in the cerebellum and in the medulla/pons, where the density of sites was one quarter that observed in the hippocampus.

To determine if the binding sites for [³H]-D-AP5 were identical with NMDA receptors characterized in electrophysiological studies, a number of excitatory amino acid antagonists of known pharmacological profile were tested as inhibitors of binding. Within an homologous series of ω -phosphono- α -aminocarboxylates², both ($\pm$)-AP3 and ($\pm$)-AP4, which are the ω -phosphono analogues of aspartate and glutamate respectively, were relatively ineffective as inhibitors of binding, whereas the longer chain analogue, ($\pm$)-AP7, was almost equipotent with ($\pm$)-AP5.

Table 1 Inhibition of [3 H]-D-AP5 binding to cortical membranes by excitatory amino acid agonists and antagonists

Antagonists	K_i (μ M)
($\pm$)-2-Amino-3-phosphonopropanoate (AP3)	560
($\pm$)-2-Amino-4-phosphonobutanoate (AP4)	260
($\pm$)-2-Amino-5-phosphonopentanoate (AP5)	1.7
($\pm$)-2-Amino-6-phosphohexanoate (AP6)	42
($\pm$)-2-Amino-7-phosphonoheptanoate (AP7)	2.4
($\pm$)-2-Amino-8-phosphono-octanoate (AP8)	640
D(-)-AP5	0.62
L(+)-AP5	55
γ -D-Glutamylglycine (γ -D-GG)	40
($\pm$)- <i>cis</i> -2,3-Piperidine dicarboxylate (<i>cis</i> -2,3-PDA)	78
γ -D-Glutamylaminomethyl sulphate (γ -D-GAMS)	770
Agonists	
NMDA	11
Quisqualate	250
Kainate	870
L-Aspartate	11
L-Cysteate	120
L-Cysteine sulphinate	13
L-Glutamate	0.9
L-Homocysteate	3.9
L-Homocysteine sulphinate	10
L-S-Sulphocysteine	2.1
Quinolinat	350
L-O-Sulphoserine	25
L-O-Phosphoserine	1,000

Membranes were incubated with [3 H]-D-AP5 (20 nM) as described in Fig. 1 in the presence of a range of concentrations of inhibitor (10 nM–1 mM). IC_{50} values were determined from log concentration/inhibition curves and K_i values calculated¹⁶. Results are mean values from two or three experiments.

The homologues ($\pm$)-AP6 and ($\pm$)-AP8 were of intermediate potency (Table 1). There was a close quantitative correlation between the potency of these compounds as inhibitors of [3 H]-D-AP5 binding and their potencies as antagonists of NMDA-induced depolarizations and of electrically evoked depolarizations in spinal cord preparations (Fig. 3). The effects of other amino acid antagonists on [3 H]-D-AP5 binding also paralleled their known potencies as NMDA antagonists^{1–3}. Thus binding was stereoselective for the D-isomer^{2,3}, L(+)-AP5 being 80-fold weaker than D(-)-AP5. The slight activity of the L form may be due to incomplete resolution, consistent with the small difference in magnitude of the optical rotations of the two isomers. In addition, γ -D-GG and *cis*-2,3-PDA, which are less potent (and less selective) antagonists for NMDA receptors than are AP5 and AP7, were of intermediate potency. By contrast, γ -D-GAMS, which antagonizes neuronal depolarizations induced by kainate and quisqualate with little or no effect on NMDA-induced responses^{13,14}, was ineffective as an inhibitor of [3 H]-D-AP5 binding (Table 1). This strongly suggests that [3 H]-D-AP5 binds to the NMDA-type receptor identified in electrophysiological studies.

Depolarizations induced by agonists such as glutamate and aspartate are composite responses mediated by different types of receptors. This, coupled with other complications, such as differential rates of amino acid uptake, makes it difficult to compare relative potencies of agonists at particular excitatory receptors in electrophysiological studies. It was, therefore, of great interest to determine affinities of a range of agonists tested as inhibitors of [3 H]-D-AP5 binding to cerebral cortical membranes. Of the three amino acids normally considered to be selective receptor agonists, NMDA was the most potent being 25 and 80 times more potent than quisqualate and kainate, respectively. (The quisqualate activity may indeed be due to a trace of glutamate detected in our preparation by high-voltage paper electrophoresis.) The other agonists listed in Table 1 have all been found in brain and/or other body tissues or fluids and can thus be considered as possible neurotransmitters. Both

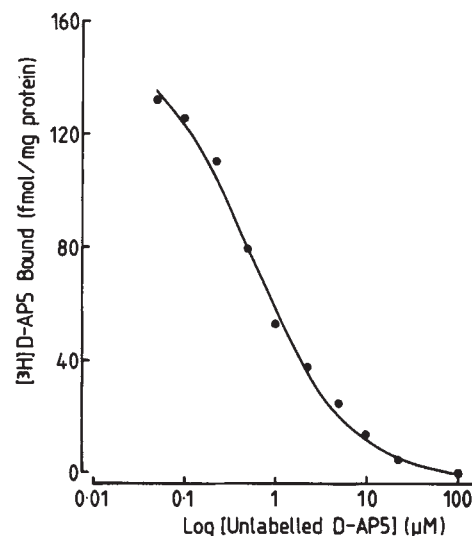


Fig. 1 Concentration dependence of [3 H]-D-AP5 binding to rat cerebral cortical membranes. Cerebral cortex from male Wistar rats was homogenized in 15 volumes ice-cold 0.32 M sucrose, pH 7, and the homogenate centrifuged at 1,000g for 10 min at 4 °C. The supernatant was recentrifuged (17,000g; 20 min) and the resultant P₂ pellet lysed with 40 volumes glass-distilled water (GDW). Following incubation at 35 °C for 30 min, the membranes were centrifuged at 50,000g for 10 min, then washed twice by resuspension in 40 volumes GDW and centrifugation (50,000g; 10 min). The final pellet was resuspended in 25 volumes 50 mM Tris-HCl buffer, pH 7.3. Binding of [3 H]-D-AP5 was measured by preincubating 0.4 ml aliquots of membrane suspension (0.3–0.5 mg protein) with 50 μ l D-AP5 (final concentration 0.05–100 μ M) or with buffer alone (control) for 3 min at 25 °C before addition of 50- μ l [3 H]-D-AP5 (27.3 Ci mmol⁻¹; custom-labelled by Amersham International). Final concentration of [3 H]-ligand was 17 nM. Samples were centrifuged after 25-min incubation at 25 °C (20,000g; 1 min), the clear supernatant aspirated and the amount of radioactivity in the pellets measured by liquid scintillation counting (Packard Tricarb 460 CD). Specific binding of [3 H]-D-AP5 was determined by subtracting the nonspecific component (binding in the presence of 1 mM unlabelled D-AP5) from the total binding. Binding in control samples was 155 fmol per mg protein. Each point is the mean of three determinations. Protein was assayed as described by Lowry¹⁷. Results were fitted by least squares¹⁸ to the logistic expression $Y = MX^P/(X^P + EC_{50}^P)$ and calculated values were M , 153 ± 9 fmol per mg protein; EC_{50} , 0.56 ± 0.11 μ M and P , 0.9 ± 0.08 .

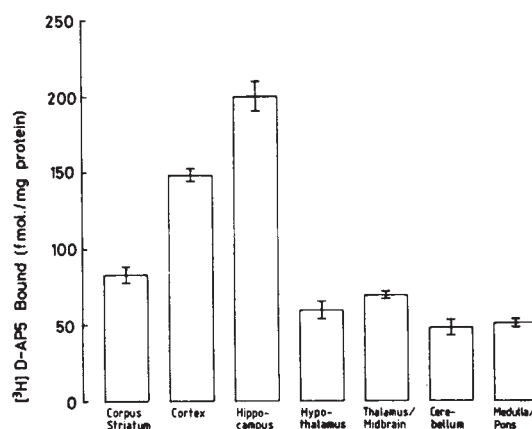


Fig. 2 Regional distribution of [3 H]-D-AP5 binding sites. Each brain area was carefully dissected as described by Glowinski and Iversen²⁰. Membranes were prepared and specific binding of [3 H]-D-AP5 (16 nM) measured as in Fig. 1. Results are mean values $\pm$ s.e. ($n = 12$) from two separate experiments.

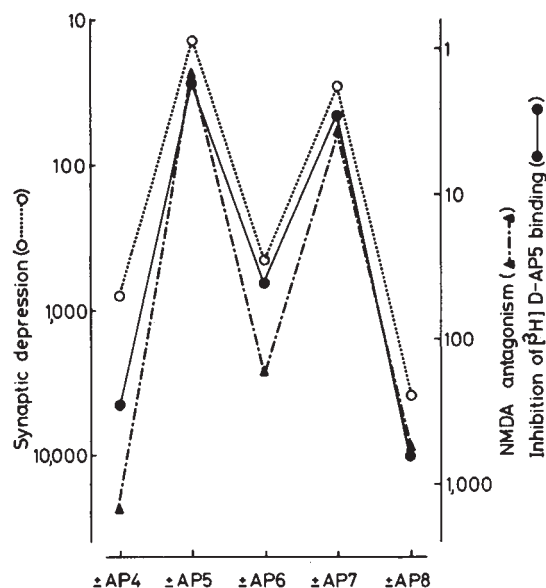


Fig. 3 Comparison of the effects of an homologous series of ω -phosphono- α -aminocarboxylates on electrically and NMDA-evoked responses recorded from ventral roots of frog spinal cord and on the binding of [3 H]-D-AP5 to rat cerebral cortical membranes. For details of electropharmacological measurements see ref. 2. [3 H]-D-AP5 binding was measured as described in Fig. 1, in the presence of concentrations of inhibitor between 10 nM and 1 mM. Left-hand ordinate scale and open circles indicate micromolar concentrations of phosphono-amino acid required to produce a 20% depression in amplitude of the pen recorded dorsal root-evoked ventral root potential. The right-hand ordinate scale and filled triangles indicate concentrations (μ M) required to produce a dose ratio of two for antagonism of NMDA-evoked responses and filled circles the K_i value (μ M) for inhibition of [3 H]-D-AP5 binding.

L-aspartate and L-Glutamate were more potent than their sulphonic and sulphonic acid analogues. In addition, all the amino acids of the glutamate chain length were more potent than aspartate and its analogues. L-Glutamate had the highest affinity of all agonists tested, being more than ten times as potent as L-aspartate, and having a K_i similar to that of D-AP5 itself. In contrast, quinolinate, a known selective NMDA type agonist⁷, had only low affinity for the D-AP5 binding site.

The findings of this study may be compared with those of another investigation in which [3 H]-($\pm$)-AP7 was used as the binding ligand¹⁵. Although there are several similarities in the results of the two studies, the conclusion¹⁵ that [3 H]-AP7 binds predominantly to quisqualate-type receptors conflicts with our findings with [3 H]-D-AP5 and, indeed, is somewhat paradoxical. Like AP5, AP7 is considered highly selective for NMDA-type receptors in electrophysiological studies, and has little or no effect on quisqualate induced depolarizations². It is possible that the nature of the AP7 binding site was transformed by the conditions used (notably the use of freeze-stored rather than fresh membranes). Alternatively, a misleading profile of binding inhibition may have been obtained by the use of the racemic form of [3 H]-AP7 rather than the pharmacologically active D(-) form of the substance¹⁹.

The high affinity of L-glutamate for [3 H]-D-AP5 binding sites found in the present work contrasts with the apparent low sensitivity of L-glutamate-induced responses to such specific NMDA receptor antagonists¹. However, the sensitivity of L-glutamate-induced responses to specific NMDA antagonists is probably masked by the actions of exogenous L-glutamate at other receptors⁵, perhaps including extrasynaptic receptors not involved in transmission processes. A role of NMDA receptors in synaptic excitation is, however, well established¹.

The present results constitute the first evidence as to the relative affinities of endogenous (and likely endogenous) excita-

tory amino acids for NMDA receptors. While relative affinities do not necessarily identify a transmitter, clearly L-glutamate must be considered a strong candidate for the transmitter acting not only at kainate/quisqualate receptors, but at NMDA receptors as well.

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Magnesium gates glutamate-activated channels in mouse central neurones

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The responses of vertebrate neurones to glutamate involve at least three receptor types¹. One of these, the NMDA receptor (so called because of its specific activation by *N*-methyl-D-aspartate), induces responses presenting a peculiar voltage sensitivity²⁻⁶. Above resting potential, the current induced by a given dose of glutamate (or NMDA) increases when the cell is depolarized⁴⁻⁶. This is contrary to what is observed at classical excitatory synapses, and recalls the properties of 'regenerative' systems like the Na⁺ conductance of the action potential. Indeed, recent studies of L-glutamate, L-aspartate and NMDA-induced currents have indicated that the current-voltage (*I*-*V*) relationship can show a region of 'negative conductance' and that the application of these agonists can lead to a regenerative depolarization⁴⁻⁶. Furthermore, the NMDA response is greatly potentiated by reducing the extracellular Mg²⁺ concentration ([Mg²⁺]_o) below the physiological level (~1 mM)^{7,8}. By analysing the responses of mouse central neurones to glutamate using the patch-clamp technique⁹, we have now found a link between voltage sensitivity and Mg²⁺ sensitivity. In Mg²⁺-free solutions, L-glutamate, L-aspartate and NMDA open cation channels, the properties of which are voltage independent. In the presence of Mg²⁺, the single-channel currents measured at resting potential are chopped in bursts and the probability of opening of the

channels is reduced. Both effects increase steeply with hyperpolarization, thereby accounting for the negative slope of the I - V relationship of the glutamate response. Thus, the voltage dependence of the NMDA receptor-linked conductance appears to be a consequence of the voltage dependence of the Mg^{2+} block and its interpretation does not require the implication of an intramembrane voltage-dependent 'gate'.

The experiments were performed on mesencephalic or striatal neurones from 13- or 15-day-old mouse embryos which were kept in culture for 6–10 days as described by Beaujouan *et al.*¹⁰. In the two patch-clamp configurations used ('whole-cell recording' and 'outside-out patches')⁹, the pipette solution usually contained (mM): CsCl, 140; NaCl, 4; HEPES-K, 10 (pH 7.2); $CaCl_2$, 0.5; EGTA 5 ($pCa = 7.3$), and the extracellular solution contained: NaCl, 140; KCl, 2.8; $CaCl_2$, 1.0; HEPES-Na, 10 (pH 7.2). Neurones used for whole-cell recordings were typically less than 12 μm in diameter with two or three fine processes. We can therefore assume that most of the cell membrane was adequately clamped in the whole-cell recording mode. The agonists, L-glutamate (referred to below as glutamate), L-aspartate, NMDA, quisqualate and kainate, were applied by bath perfusion.

In the whole-cell recording mode, when the membrane potential was held at -60 mV in a Mg^{2+} -free solution, glutamate (2 – 10 μM) elicited, in most neurones, an inward current which remained stable over periods of up to 10 min.

The effects of membrane potential and Mg^{2+} on the glutamate-activated current are illustrated in Fig. 1a. In the absence of extracellular Mg^{2+} , the glutamate-induced current varied nearly linearly with membrane potential. The reversal potential was 0 mV. The addition of extracellular Mg^{2+} (500 μM) pro-

duced little change in the outward currents, but markedly reduced the inward currents. The blocking effect of Mg^{2+} increased as the cell was hyperpolarized.

The effect of Mg^{2+} on the glutamate-induced current noise (Fig. 1b) was parallel to that observed on the total current. The power spectra of the glutamate-induced noise were well fitted by single lorentzians having a similar cut-off frequency at -40 and $+40$ mV in Mg^{2+} -free solution, and at $+40$ mV in Mg^{2+} (500 μM) (Fig. 1c). Under the usual assumptions made in the spectral analysis of transmitter-induced currents¹¹, this indicates that the mean open time of the channels opened by glutamate in Mg^{2+} -free solutions is independent of membrane potential. This open time is nearly 5 ms at $27^\circ C$ (Fig. 1c) and 3 ms at $30^\circ C$ (Fig. 1b). It is not altered by Mg^{2+} (1 mM) in the positive potential range.

We repeated the experiments described above on outside-out patches. In Mg^{2+} -free solutions, glutamate (1 – 10 μM) activated a homogeneous population of channels. With CsCl-containing pipettes, the elementary current varied linearly between -80 mV and $+40$ mV, inverting around 0 mV. The value of the single-channel conductance was 49 ± 8 pS ($n = 14$). When CsCl in the pipette was replaced by KCl, the analysis at potentials above -40 mV became difficult due to the currents flowing through the voltage-dependent K^+ channels. Between -80 mV and -40 mV, however, the glutamate-induced single-channel currents were identical to those measured with CsCl inside the pipette. In one experiment, external Cl^- was replaced by isethionate and the elementary current through the glutamate-activated channels was unchanged. These results suggest that the channels opened by glutamate are cation channels discriminating only slightly between monovalent cations, and

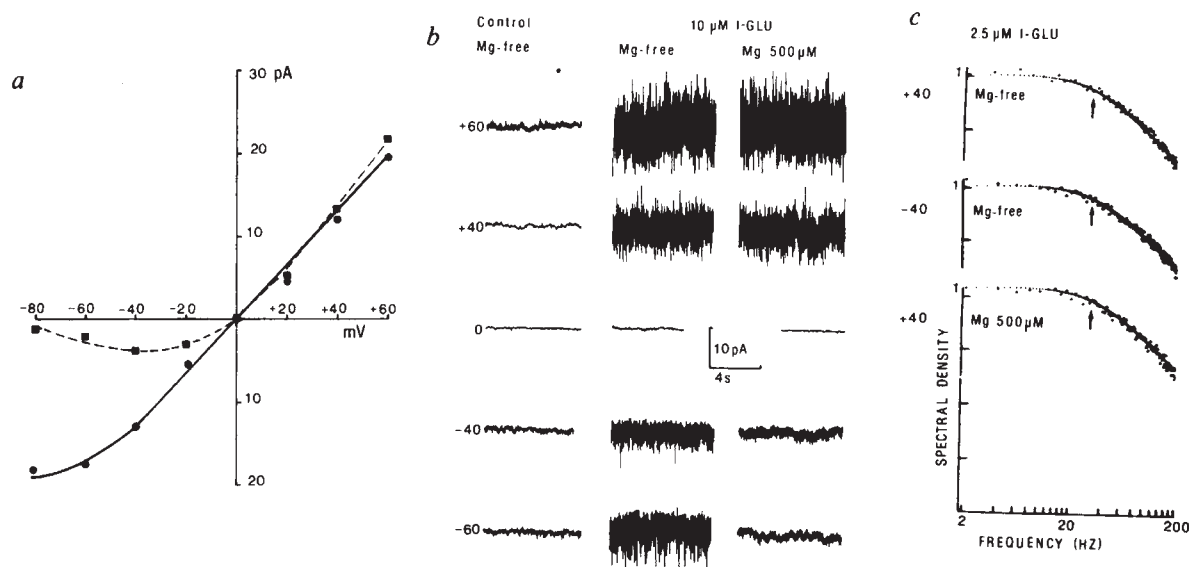


Fig. 1 Glutamate-induced currents in the whole-cell recording mode. **a**, Voltage dependence of the glutamate-induced current. The glutamate-activated current was calculated at each potential as the difference between the current measured in control (Mg^{2+} -free or 500 μM Mg^{2+}) solutions and after addition of glutamate (10 μM). In a Mg^{2+} -free solution ($\bullet$), the I - V relationship is approximately linear between $+60$ and -60 mV. The bend observed below -60 mV was not observed in all experiments, although in many cases the slope of the I - V relation was smaller in the negative potential range than in the positive range. In a solution containing Mg^{2+} (500 μM) ($\blacksquare$), the currents are reduced at negative potentials, and the I - V relationship shows a 'negative resistance' region. In the positive potential range, no significant change is observed. **b**, Glutamate-induced current noise. Same experiment as in **a**. The d.c. shift of the current is not illustrated. The control traces shown in the left-hand column were taken in Mg^{2+} -free solution in the absence of glutamate. The records of the central column were taken after the addition of glutamate (10 μM) (Mg^{2+} -free solution), and the records on the right were taken in a solution containing Mg^{2+} (500 μM) and glutamate (10 μM). The control records for 500 μM Mg^{2+} solutions (not shown) were identical to those shown for Mg^{2+} -free. **c**, Power spectra of glutamate-induced current noise. In this experiment, the concentration of glutamate was 2.5 μM . Each spectrum was obtained by subtraction of control spectra from spectra recorded in the presence of glutamate. Spectral density: arbitrary units, log scale. The cut-off frequencies are: 34.5 Hz (upper record), 31.8 Hz (middle record) and 32.5 Hz (lower record). These frequencies correspond to respective time constants of 4.6 , 5.0 and 4.9 ms. Temperature: $30^\circ C$ (**a**, **b**); $24^\circ C$ (**c**).

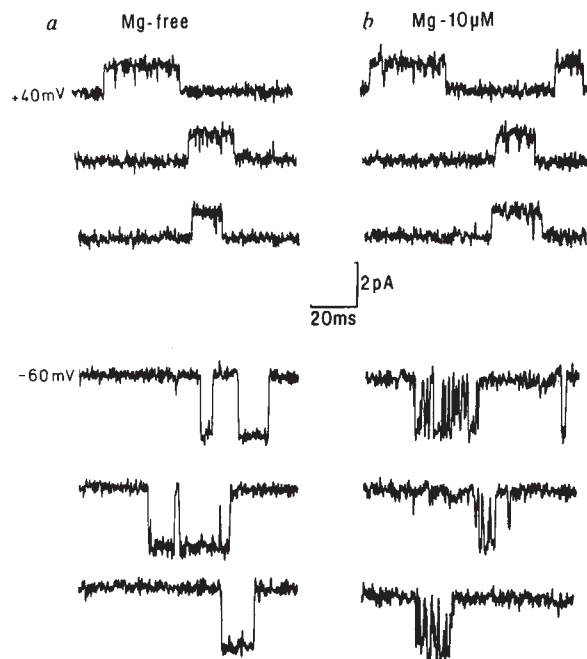


Fig. 2 Glutamate-induced single-channel currents in outside-out patches. In Mg^{2+} -free solution (a), the single-channel currents appear as simple events on both sides of the reversal potential (about -5 mV in this experiment). After addition of Mg^{2+} ($10 \mu\text{M}$) (b) the currents recorded at $+40$ mV are unchanged whereas the currents recorded at -60 mV appear as bursts. The size of the single-channel current is not altered by the addition of $10 \mu\text{M}$ Mg^{2+} . At higher Mg^{2+} concentrations (50 – $100 \mu\text{M}$) an apparent reduction of the elementary current was observed (not shown), but this probably only reflects the inability of the recording system to display faithfully the transient openings of the channels. In this experiment, MgCl_2 (2 mM) had been added to the internal solution. Note that the single-channel conductance is 51 pS at -60 mV, and only 33 pS at $+40$ mV. This discrepancy was observed in all experiments where Mg^{2+} (2 mM) was present in the internal solution but was not seen when Mg^{2+} was absent. Thus, when Mg^{2+} was absent both from the internal and external solutions, the I – V relationship of the glutamate-induced current was linear (between -60 and $+60$ mV) at the single-channel level as well as (Fig. 1) at the whole-cell level.

resembling in this respect the glutamate-activated channels of the insect neuromuscular junction^{12,13}.

The open-time distribution of the single-channel currents could be reasonably well fitted by a single exponential with a voltage-independent time constant. Finally, the frequency of occurrence of the channel openings was (in a given patch) not noticeably affected by membrane potential. It thus appears that in Mg^{2+} -free solution, the three 'elementary' properties of the individual channels (elementary conductance, mean open time and frequency of opening) are, to a first approximation, voltage independent.

In the presence of extracellular Mg^{2+} ($10 \mu\text{M}$) the single-channel currents observed at positive potentials were identical to those recorded in the absence of Mg^{2+} , but at negative potentials major modifications were observed. The most striking change (Fig. 2) was that each channel opened in a burst, in a pattern similar to that first observed by Neher and Steinbach¹⁴ in their study of the effects of local anaesthetics on acetylcholine-induced currents.

The appearance of such bursts suggested that extracellular Mg^{2+} may be acting by a 'fast channel block' mechanism^{14–18}. We attempted to test some of the predictions of the simplest form of this scheme, in which Mg^{2+} ions block only the open state. If we call $\bar{t}_o$ the mean open time of a channel, $\bar{t}_b$ the mean duration of the group of openings constituting a burst, and $\bar{t}_c$ the mean value of the gap duration (blocked time) inside a burst^{14,18} (see insert, Fig. 3), the fast channel block scheme

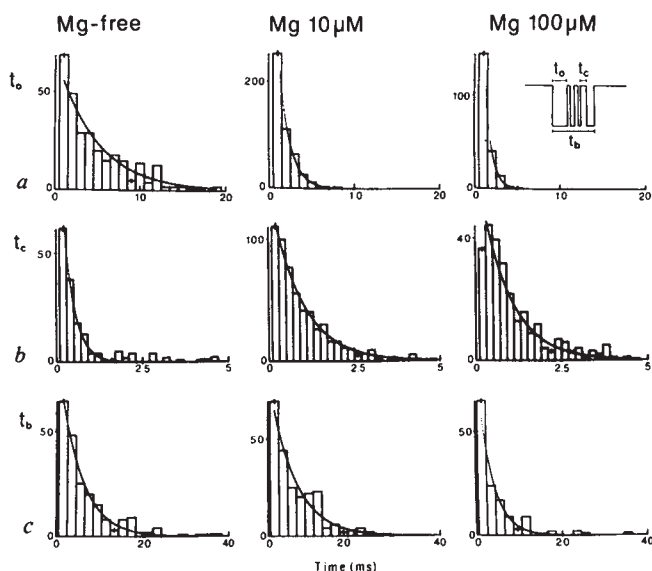


Fig. 3 Histograms of time intervals between open and closed states. Same experiment as in Fig. 2. Data recorded at -60 mV and analysed as described by Trautmann and Siegelbaum²³. Bin length: 1 ms (a), 0.2 ms (b), 2 ms (c). Ordinate: number of observations. The insert (upper right) shows examples of open time (t_o), short closed time (t_c) and burst duration (t_b) used for analysis. a, Distribution of open times (t_o). The mean open time, $\bar{t}_o$, measured as the time constant of the exponential fitted on the histogram, was 4.7 ms in Mg^{2+} -free solution, 1.2 ms in $10 \mu\text{M}$ Mg^{2+} , and 0.7 ms in $100 \mu\text{M}$ Mg^{2+} . b, Distribution of short closed times (t_c). The values plotted are the durations of the short closures (gaps) inside the bursts illustrated in Fig. 2. The mean value, $\bar{t}_c$, was 0.3 ms in Mg^{2+} -free solution, 0.88 ms in $10 \mu\text{M}$ Mg^{2+} , and 0.84 ms in $100 \mu\text{M}$ Mg^{2+} . The last two values are comparable, as expected from a simple fast channel block model, in which they would imply a value close to $1,100 \text{ s}^{-1} (1/\bar{t}_c)$ ^{14,18} for the rate constant of dissociation of Mg^{2+} from the channel. The $\bar{t}_c$ values in Mg^{2+} -free correspond to rare events, one of which is illustrated in Fig. 2a, middle record at -60 mV, Mg^{2+} -free. The gaps observed in such 'control' conditions were not analysed in detail, but their frequency and mean duration were similar at -60 mV and at $+40$ mV (not shown). c, Distribution of burst durations (t_b). The burst durations were measured by the same method as the open time, except that gaps of less than 5 ms (instead of gaps of 0.5 ms in the case of open times) were ignored. The time constant of the exponential, $\bar{t}_b$, was 5.7 ms in Mg^{2+} -free solution, 4.9 ms in $10 \mu\text{M}$ Mg^{2+} , 3.2 ms in $100 \mu\text{M}$ Mg^{2+} . The difference between $\bar{t}_o$ and $\bar{t}_b$ in Mg^{2+} -free solutions corresponds to the presence of the few gaps mentioned above.

predicts that as the Mg^{2+} concentration, $[\text{Mg}^{2+}]_o$, increases, $\bar{t}_o$ should decrease while $\bar{t}_c$ should remain constant. These two predictions were verified in the experiment illustrated in Fig. 3. However, the simple sequential scheme also predicts that, at low Mg^{2+} concentrations, the burst duration should increase when $[\text{Mg}^{2+}]_o$ increases, whereas the total charge carried during a burst should remain constant (see also ref. 19). This prediction was not verified. As illustrated in Fig. 3, the burst duration decreased when $[\text{Mg}^{2+}]_o$ was increased, and this effect was enhanced by hyperpolarization (not shown).

That a simple fast-channel block model cannot account for all the blocking effects of Mg^{2+} is further indicated by the fact (Fig. 4) that Mg^{2+} reduces the frequency of opening of the glutamate-induced channels. This effect and the reduction of burst duration (illustrated in Fig. 3) may well be the result of a common underlying mechanism, for example, slow channel block²⁰ or increased desensitization²¹. In general, it is premature to propose any specific scheme but it seems clear that the blocking effect of Mg^{2+} involves at least two voltage-dependent processes, one accounting for the fast flickering of the single-channel current, the other explaining the reduced frequency of

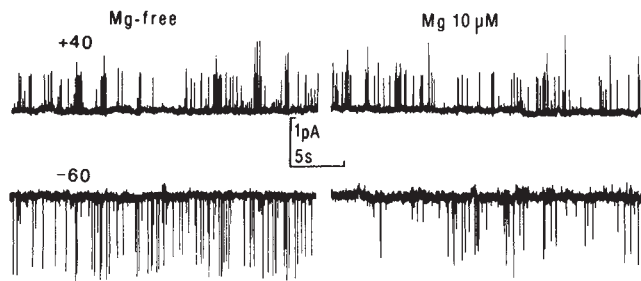


Fig. 4 Glutamate-induced currents in outside-out patches. Same experiment as in Fig. 2. The records, taken at slow speed on a chart recorder, illustrate the reduction in the frequency of channel openings produced by Mg^{2+} ($10 \mu M$) at -60 mV (lower records), whereas no change in frequency is observed at $+40$ mV (upper records). The small size of the elementary currents, and the apparent reduction of their size by Mg^{2+} (-60 mV) both result from the filtering introduced by the chart recorder and were not present when the currents were displayed on an oscilloscope (Fig. 2).

channel openings. A similar dual block has been recently proposed for the Na^+ block of some K^+ channels²².

The hypothesis that the Mg^{2+} -sensitive glutamate-activated channels are of the NMDA type was supported by the finding that, both in the whole-cell recording mode and in outside-out patches, NMDA (2 – $10 \mu M$) and L-aspartate ($10 \mu M$) elicited responses similar to those induced by L-glutamate, while kainate (up to $10 \mu M$) and quisqualate (up to $5 \mu M$) were ineffective.

Thus, our observations provide an explanation for the ionic mechanism of the glutamate response associated with NMDA receptors. Whereas early observations had suggested that glutamate may act through a K^+ conductance decrease³, more recent work has indicated that L-glutamate, L-aspartate and NMDA all induce a voltage-dependent conductance increase^{4–6}. Our data confirm this interpretation and further indicate that the channels opened by glutamate are permeant to both Na^+ and K^+ . In addition, they suggest that the voltage dependence of the conductance does not result from a direct effect of the membrane potential on the receptor-channel complex but rather is the consequence of a voltage-dependent Mg^{2+} blockade.

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Voltage-gated K^+ channels in human T lymphocytes: a role in mitogenesis?

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Membrane receptors and ion transport mechanisms probably have an important role in lymphocyte activation leading to T-lymphocyte proliferation in the immune response. Here we have applied a gigohm-seal patch clamp technique¹ to reveal the identity and properties of ion channels in human T lymphocytes. A voltage-dependent potassium channel bearing a resemblance to the delayed rectifier of nerve and muscle cells was found to be the predominant ion channel in these cells. In the whole cell recording conformation, the channels open with sigmoid kinetics during depolarizing voltage steps, reaching a maximum K^+ conductance of 3–5 nS. The current subsequently becomes almost completely inactivated during a long-lasting depolarization. Currents through single K^+ channels recorded in whole cell and outside-out patch recording conformations reveal a unitary channel conductance of about 16 pS in normal Ringer solution. Thus, the peak current corresponds to approximately 200–300 conducting K^+ channels per cell. Phytohaemagglutinin (PHA), at concentrations that produce mitogenesis, alters K^+ channel gating within 1 min of addition to the bathing solution, causing channels to open more rapidly and at more negative membrane potentials. ³H-thymidine incorporation by T lymphocytes following PHA stimulation is inhibited by the 'classical' K^+ channel blockers tetraethylammonium and 4-aminopyridine, and also by quinine, at doses found to block the K^+ channel in voltage-clamped T lymphocytes, suggesting that K^+ channels may play a part in mitogenesis.

Figure 1 shows outward potassium currents recorded from a human T lymphocyte in the whole cell conformation. Depolarizing voltage steps up to -40 mV given from a holding potential of -80 mV produce very small leakage currents, representing an input resistance of greater than $20 G\Omega$ for this cell. At -30 mV, outward current is just noticeable, and larger depolarizing pulses produce larger outward currents as more channels open. The current is carried by K^+ ions from the cytoplasm in diffusional equilibrium with the pipette solution. If the extracellular K^+ concentration is raised to the concentration within the pipette (not shown), we observe inward K^+ current below a well defined reversal potential near 0 mV. During a depolarizing pulse, the voltage-gated K^+ channels open after an initial delay and then slowly become inactivated. The inactivation process most closely resembles the K^+ channel inactivation described in molluscan nerve cell bodies² displaying 'cumulative inactivation', as shown in the inset of Fig. 1. Here, two identical voltage pulses to 0 mV are given, separated by 50 ms. The slow decline of current during the first pulse is interrupted by repolarization, and during the second pulse current rises to a lower level than at the end of the first pulse. This phenomenon has been modelled in a kinetic scheme with two separate inactivated states for the channel³.

The K^+ channel inactivation process has two consequences for our experiments. First, because recovery from inactivation is very slow, pulses delivered faster than every 20 s result in decreasing current magnitude during a pulse series. We have found that replacement of chloride by fluoride in the pipette solution helps to provide the stability required at low pulse frequencies, allowing recording over a period of hours with little rundown. Second, because inactivation is nearly complete at depolarized potentials, it is possible to record current through single channels in the whole cell conformation, as illustrated in

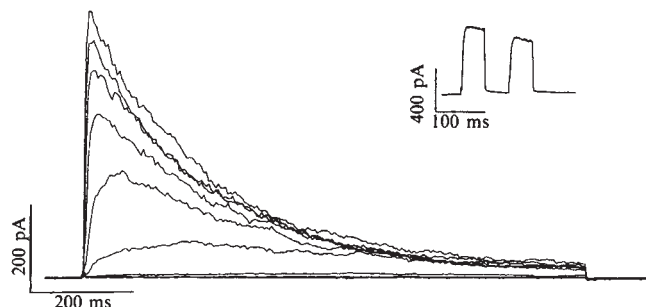


Fig. 1 Currents recorded from a T lymphocyte in the whole cell conformation. Peripheral blood was obtained from healthy volunteers (initials given in figure legends), and T lymphocytes were separated as described previously²⁹. The membrane potential was held at -80 mV, and depolarizing pulses were given every 20 s in 10 mV steps up to $+30$ mV. Currents were recorded with a 10 G Ω feedback resistor, with active lowpass filtering at 2 kHz. Cell from S.C. Inset: a K⁺ current record illustrating cumulative inactivation. Twin depolarizing pulses to 0 mV from a holding potential of -80 mV. Cell from S.G. T lymphocytes were suspended in medium RPMI-1640 supplemented with 10% human AB serum, 25 mM HEPES, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and 2 mM L-glutamine (Gibco), and maintained at 37 °C in humidified 5% CO₂ atmosphere. In three separate determinations the cell population studied contained more than 90% T lymphocytes, estimated by Leu-1 (Pan T, Becton-Dickinson) monoclonal antibody binding and fluorescence activated cell sorter analysis. All voltage clamp measurements were made at room temperature (20–22 °C). The pipette input resistance, usually 3–6 M Ω , was measured with the pipette in the bath, and was used to set the series resistance compensation. The cells were bathed in a mammalian Ringer solution containing (in mM) 160 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂ and 5 HEPES, at pH 7.4. The pipette contained an 'internal' solution consisting of 140 KF, 1 CaCl₂, 2 MgCl₂, 11 K₂EGTA and 10 HEPES, pH 7.2 (free Ca²⁺ concentration less than 2×10^{-9} M, based on a solubility product of 3.4×10^{-11} M³ for CaF₂³⁰). High resistance seals (often 50–200 G Ω) sometimes formed spontaneously on touching the pipette to the cell surface, but more often after application of a small negative pressure (usually <20 cm H₂O). Whole cell recording was achieved by applying strong negative pressure to the pipette interior (more than 100 cm H₂O) in order to disrupt the membrane patch within the pipette.

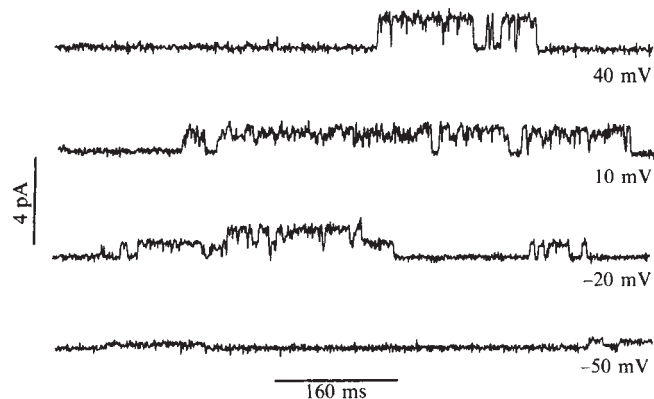


Fig. 2 Single K⁺ channel currents recorded from a T lymphocyte in the whole cell conformation. Same bathing and pipette solutions as Fig. 1. Membrane potential held at potentials indicated. 800 Hz filter. Cell from S.G.

Table 1 Viability of T lymphocytes exposed to K⁺ channel blockers

Expt	Treatment	% Viability (Trypan)	³ H-thymidine (c.p.m. $\pm$ s.d.)
I	Control	83	17,000 $\pm$ 5,000
	4AP+PHA	87	19,700 $\pm$ 9,100
II	Control	95	51,400 $\pm$ 2,700
	TEA+PHA	92	48,200 $\pm$ 4,800
III	Control	100	73,600 $\pm$ 3,400
	Quinine+PHA	94	53,400 $\pm$ 9,300

T lymphocytes (2×10^6 ml⁻¹) were incubated in sterile 12 $\times$ 75 mm tubes in medium described in Fig. 1 at 37 °C in humidified 5% CO₂ in the presence of 50 μ g ml⁻¹ PHA and 8.6 mM 4AP, 20 mM TEA or 100 μ M quinine, for 48 h. Control T lymphocytes were suspended in medium alone for the same duration. Cells were then washed thrice in Hank's balanced salt solution supplemented with 25 mM HEPES, resuspended in media at 10^6 cells ml⁻¹ and viability assessed by Trypan blue dye exclusion. The cells were then cultured in microtitre plates (10⁵ cells per well) and incubated with 8 μ g ml⁻¹ PHA, and ³H-thymidine uptake was measured 64 h later. Cells were from M.T. (I), N.W. (II) and K.C. (III). Viability in the presence of 4AP and TEA was assessed in three additional determinations by Trypan blue dye exclusion with similar results. All concentrations given are final concentrations.

Fig. 2. When the membrane potential is stepped and held at a depolarized level, K⁺ current is first turned on and then becomes inactivated nearly to zero, with single channel events seen above the background noise. From records such as these, and from outside-out recordings in which the patch is excised from the cell (not shown), we estimate a single channel conductance of about 16 pS in normal Ringer. The single channel currents are 'flickery' and tend to become inactivated with rapid pulsing, whether recorded from intact cells on first making a seal, from whole cells as in Fig. 2, or in outside-out patches. Dividing the mean maximum whole cell conductance, 4 nS, by the single channel conductance, provides an estimate of at least 250 conducting K⁺ channels per cell, although more channels may be present than those opening during a large depolarization.

In a small number of cells, whole cell recording revealed qualitatively different outward currents that slowly become activated at potentials above 0 mV and fail to become inactivated. This minority population of cells may represent contaminating non-T cells that are present in the T-lymphocyte-enriched population at a frequency of about 10%. In more than 90% of the cells studied, the voltage-gated K⁺ channel described here was clearly dominant in the recordings. Our recording conditions, with very low free calcium concentration and fluoride in the pipette, may have prevented our seeing other channels that might be present in intact T lymphocytes, such as calcium channels reported to be present in a B-cell myeloma line⁴ or calcium-activated channels. Calcium currents in snail neurones are abolished by internal perfusion with fluoride-containing

solutions⁵. The K⁺ channel described here differs from calcium-activated 'maxi-K⁺' channels described previously in other tissues^{6,7} in its calcium sensitivity, gating properties, single channel conductance and pharmacological sensitivity. The free Ca²⁺ in our pipette solution was only 2 nM (see Fig. 1 legend), whereas 1–10 μ M internal Ca²⁺ is required to activate Ca²⁺-dependent 'maxi-K⁺' channels with the voltage dependence found in the present work^{8–10}. We have not investigated the possible dependence of the K⁺ channel on intracellular free Ca²⁺ concentration. Sigmoid activation kinetics and voltage-dependent inactivation are characteristic of delayed rectifier K⁺ channels, but not Ca²⁺-activated K⁺ channels. The ubiquitous maxi-K Ca²⁺-activated channels have a very large unitary conductance, 100–240 pS⁶, although Ca²⁺-activated K⁺ channels of 20–50 pS, closer to the 16 pS found here, have been reported in *Helix* neurones^{11,12} and in red blood cells¹³, but have not yet been well characterized. Pharmacological experiments provide additional characterization of the voltage-gated K⁺ channels. T-lymphocyte K⁺ channels are blocked reversibly in a dose-dependent manner by tetraethylammonium (TEA), 4-aminopyridine (4AP) and quinine. Figure 3a illustrates dose-response curves for channel block by these three agents. The curves represent a one to one drug molecule-receptor relationship, fitted by eye to the measured fractional current remaining at various drug concentrations. Quinine is the most potent, with an inhibitory constant, K_i, of

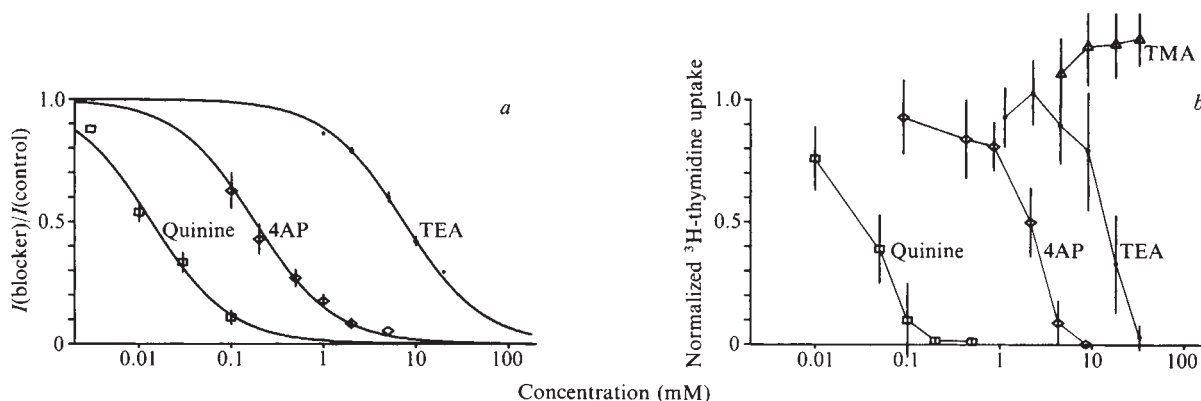


Fig. 3 Dose-response curve for blockade of the K⁺ channel (a) and inhibition of mitogenesis (b) by quinine (□), 4AP (◇) and TEA (●) in T lymphocytes. Also shown in (b) are data for tetramethylammonium (TMA, △), which did not appreciably block K⁺ currents. a, Data points show the mean K⁺ current remaining in the presence of the indicated concentration of blocker added to Ringer solution. Cells from S.G., S.B., E.S. and M.T. PHA, at concentrations used for mitogenesis, seemed to have no effect on blocking by TEA or 4AP, and for these blockers data in the presence and absence of PHA have been pooled. s.e. bars are shown for means including data from three or four cells. In all experiments the membrane potential was held at -80 mV and stepped briefly to +20 mV every 30 s. After leak subtraction the peak current in the presence of blocker was normalized to the average of control currents recorded before addition and after washout of blocker. The bath temperature was monitored with a thermistor, and current records were temperature matched to within 0.2 °C. The preparation was allowed to equilibrate in each test solution until the peak current amplitude appeared to have reached steady-state. In 4AP-containing solutions both block and recovery progressed more slowly than in quinine or TEA-containing solutions, and about 5 min was allowed before recording. Recovery from the first application of 4AP to a cell was about 90% complete, so data from the first exposure to 4AP were not included. The curves express a bimolecular interaction between blocker and channel, with the fractional current remaining expressed as $1/(1 + (B/K_i))$, where B is the drug concentration and K_i is the equilibrium dissociation constant. The K_i adjusted by eye to fit the data points was 0.014 mM for quinine, 0.19 mM for 4AP and 8 mM for TEA. b, Cells from S.C., N.W., G.C., S.G., K.L., S.B. and M.T. 10⁵ T lymphocytes per well in medium (Fig. 1 legend) were plated in 96-well round-bottomed microtitre plates (Corning) and incubated in the presence of PHA at a final concentration of 8 µg ml⁻¹, with or without quinine, 4AP or TEA at 37 °C in humidified 5% CO₂ for 64 h. Cells were pulsed with ³H-thymidine (1 µCi per well) for the final 16 h. Stock solutions of blockers in medium were titrated to pH 7.2 and added to the cells to the appropriate final concentration. All experiments were done in triplicate. The data points show the mean and s.d. from three or four experiments, of ³H-thymidine incorporation normalized to that in PHA-treated cells in the absence of blocker. Background counts from cells with no PHA added were always less than 200 c.p.m.

about 14 µM, followed by 4AP at 190 µM and TEA at 8 mM. Quinine is believed to block Ca²⁺-activated K⁺ channels, but this block is not specific¹⁴. In neuroblastoma cells, for example, quinine blocks both Ca²⁺-activated and voltage-gated K⁺ channels¹⁵. TEA also blocks both types of K⁺ channels, whereas block by aminopyridines appears to distinguish between voltage-gated and Ca²⁺-activated K⁺ channels⁷. Thus, the K⁺ channel described here seems to be distinct from any Ca²⁺-activated channel described to date, but closely resembles voltage-gated delayed rectifier K⁺ channels.

Phytohaemagglutinin is a potent mitogen for T lymphocytes, stimulating cell division by mechanisms that have been intensively studied¹⁶⁻¹⁹. Involvement of ionic channels at an early stage of PHA activation is suggested by reports of membrane depolarization²⁰, hyperpolarization^{20,21}, increased ion fluxes across the membrane^{22,23} and increased intracellular free calcium^{21,24}. We therefore studied the effects of the three K⁺ channel blockers described above and found them to inhibit PHA-induced mitogenesis in a dose-dependent manner (Fig. 3b), with the same potency sequence as for channel block (Fig. 3a). These data suggest that mitogenesis is suppressed when some fraction (perhaps 50%) of the K⁺ channels do not function. The relative potency of 4AP for channel block compared with inhibition of mitogenesis seems to be greater than that of TEA or quinine. This result may be due to scatter in the data, or it might reflect relief of 4AP block by depolarization, as reported in other tissues^{25,26}, since the normal lymphocyte resting potential is substantially more positive than the -80 mV holding potential used here^{20,21,27,28}. Complete replacement of the external sodium by 160 mM tetramethylammonium (TMA), an analogue of TEA, produced very little reduction of K⁺ currents (data not shown). Up to 33 mM TMA added to the medium did not inhibit mitogenesis, showing that the inhibition by TEA was not due to the higher osmolarity of the medium. Table 1 shows that T lymphocytes incubated for 48 h in the presence of PHA and 4AP, TEA or 100 µM quinine were viable, as assessed

by Trypan blue dye exclusion and by the ability to respond normally to added PHA after washing. Viability was reduced by 200 µM quinine. 4AP did not nonspecifically prevent ³H-thymidine uptake and incorporation because 4AP added at the time of thymidine addition, 48 h after PHA activation, did not alter thymidine incorporation.

PHA may bind to cell-surface glycoproteins, producing effects by altering membrane transport properties. Since the voltage-gated K⁺ channel described above is the predominant ion channel in our patch clamp experiments, we studied the influence of PHA on the kinetics of K⁺ channel gating. Figure 4 illustrates the major immediate effects of PHA on the K⁺ channel. In this cell before PHA addition (Fig. 4a), K⁺ current was just noticeable during depolarizing pulses to -20 mV. At more positive potentials more channels open. Shortly after adding 3 µg ml⁻¹ PHA (Fig. 4b), current was noticeable at -40 mV, about 20 mV more negative than the control. As shown in Fig. 4c the conductance-voltage relationship appears steeper and shifted towards hyperpolarized potentials after PHA treatment, with little or no change in the maximum conductance. Taking the potential required to activate half-maximal conductance as a measure and pooling the data for eight cells at PHA concentrations of 3-100 µg ml⁻¹, PHA produced a hyperpolarizing shift of -15 ± 3.5 mV (mean $\pm$ s.e.m.), with larger PHA concentrations tending to produce larger shifts. At a given potential the channels opened more rapidly following addition of PHA, causing a decrease in the time to peak current, also seen in Fig. 4. The effect of PHA was not readily reversed by washing with normal Ringer. The primary action of PHA may be to increase the voltage-dependent rate constants that lead to K⁺ channel opening. If PHA acts by altering external surface charges 'sensed' by channel activation gates, our results indicate that the local surface potential would be more negative after PHA treatment. As a result of the alteration of K⁺ channel gating by PHA more K⁺ channels would be open on the average, possibly contributing to the hyperpolarization and increased K⁺ fluxes previously

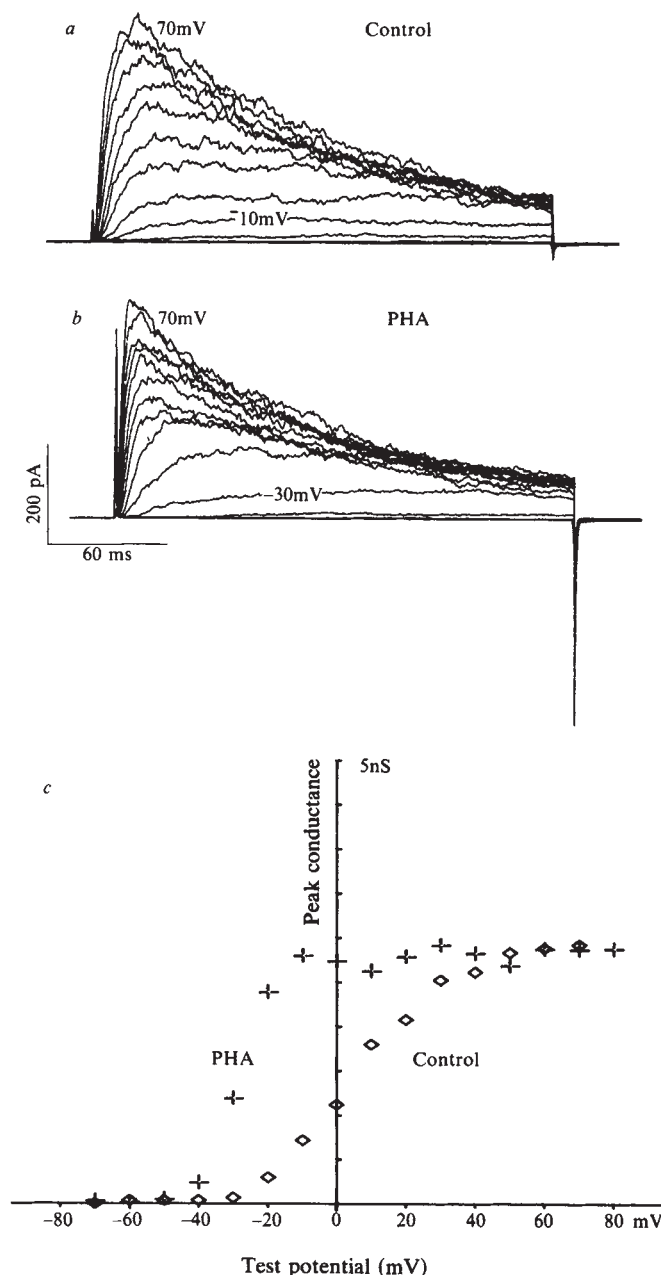


Fig. 4 *a* and *b* show whole cell currents recorded from a T lymphocyte from S.G. Same bathing and pipette solutions as previous figures, except $3 \mu\text{g ml}^{-1}$ PHA (PHA-P, Difco) was added before recording currents in *b*. PHA solutions were mixed fresh daily from aliquots of frozen 10 mg ml^{-1} stock solution. Same cell as Fig. 2. Depolarizing steps up to $+70 \text{ mV}$ in 10 mV increments were given every 20 s from the holding potential of -80 mV . *c*, The corresponding conductance-voltage relationships calculated by dividing the peak K^+ current at each depolarizing voltage step by the potential minus the measured reversal potential, -82 mV .

reported²¹⁻²⁴. The rapid effect of PHA on K^+ channel gating, taken together with the inhibition of ^3H -thymidine incorporation by K^+ channel blockers, suggests that functioning K^+ channels altered by PHA may bring about a sequence of cellular and molecular events leading to DNA synthesis.

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K channels in T lymphocytes: a patch clamp study using monoclonal antibody adhesion

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Ionic fluxes are thought to be involved in mediating the proliferation of peripheral blood lymphocytes (PBLs) in response to mitogenic substances^{1,2}. Among the earliest events occurring after the addition of mitogen to cultured lymphocytes are changes in rates of cation transport³⁻⁹. We were interested, therefore, in the possible role of ion channels in mediating the lymphocyte proliferative response. The development of patch clamp techniques by Neher and colleagues¹⁰⁻¹² has made it possible to study membrane conductances in a variety of small cell types¹². We have developed a method which uses monoclonal antibodies to make cells adhere to solid surfaces for two major reasons: (1) it is much easier to patch clamp stationary cells, and (2) the method can be used to selectively adhere a particular cell type from a heterogeneous population. We have used these techniques here to identify whole-cell potassium currents, in lymphocytes recognized by OKT11 monoclonal antibody, which are increased 1.9-fold by mitogenic stimulation.

Subclasses of human T lymphocytes, of $\sim 7 \mu\text{m}$ diameter, were selectively adhered to solid surfaces (plastic Petri dishes and glass coverslips) using monoclonal antibodies of known specificity^{13,14}. Human T-lymphocyte populations have been defined by the presence of antigenic determinants on the cell surface. Using flow cytometry we found that 75% of the cells in the monocyte-depleted PBL suspension were T lymphocytes recognized by monoclonal antibody OKT3 (Table 1). Of these T cells, 66% were predominantly helpers recognized by OKT4 and 30% were predominantly suppressor recognized by antibody OKT8.

To demonstrate selective adhesion, we used both indirect and direct immunofluorescence microscopy, the former being more sensitive than the latter. In all experiments, suspensions of PBLs

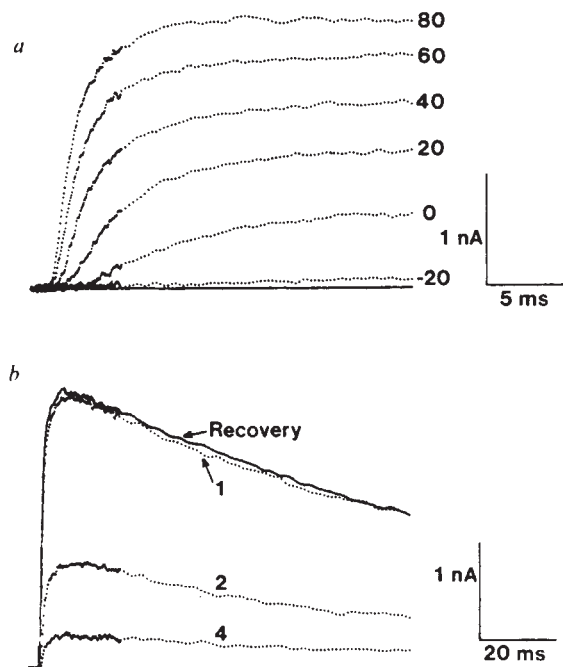


Fig. 1 Voltage-dependent outward currents in T lymphocytes. *a*, When lymphocytes were depolarized they generated an outward current. These currents were elicited by 25-ms step depolarizations to the indicated voltages from a holding potential of -70 mV. The currents in *b* were generated by 100-ms depolarizations to +100 mV. Dotted traces labelled 1, 2 and 4 resulted from first, second and fourth pulses, respectively, of a train delivered at 1.5 Hz, and the solid trace was recorded 74 s after the train. For this experiment, cells were attached with OKT11 and had been stimulated with succinyl concanavalin A for 20 h.

Methods: Patch electrodes, of 2–5 MΩ resistance, were made with borosilicate glass and filled with the following solution (mM): 140 KCl, 2 MgCl₂, 10 EGTA and 10 HEPES (pH 7.3). Just before recording, the solution bathing the antibody-adhered cells was changed to a protein-free solution of the following composition (in mM): 130 NaCl, 10 CaCl₂, 2 MgCl₂, 5 KCl and 10 HEPES (pH 7.3). After obtaining a gigaseal against the cell, further suction was applied to rupture the patch of membrane under the electrode tip. In these conditions, the electrode monitors current through the entire cell surface. The electrode was connected to a current-to-voltage (*I*-*V*) converter and the analog current signal was acquired with an LSI 11/23 computer at 50 kHz and stored for later analysis. Linear components of capacitive and ionic current were subtracted out with a P/2 procedure²⁶. Cells were voltage-clamped by controlling the positive input of the *I*-*V* converter.

were first depleted of monocytes by adhering these sticky cells to T150 tissue culture flasks (final monocyte content ~1%). For indirect immunofluorescence measurements, lymphocytes were incubated with either OKT3, OKT4 or OKT8 (Ortho Pharmaceutical Corp.), washed, made to adhere to goat anti-mouse IgG (Tago) coated surfaces, then incubated with fluorescein-labelled Tago. In each case, almost all cells adhering to the surfaces were fluorescent. The following results were obtained in control experiments: (1) in the absence of antibodies, lymphocytes did not adhere to Tago-coated surfaces; (2) lymphocytes were not fluorescently labelled by incubation in suspension with fluorescein-Tago alone; and (3) Tago is necessary for adhesion of cells that have been labelled with monoclonal antibody. These results demonstrate that monoclonal antibody is necessary in order to observe fluorescence and adhesion, and therefore the specificity of the antibody imparts specificity to the cell attachment. Direct immunofluorescence measurements, in which adherent lymphocytes were incubated with OKT3, OKT4 or OKT8 (fluorescein-labelled) and then aliquoted onto Tago-coated surfaces, showed enhancement of the specific cell population on the surface, compared with its distribution in

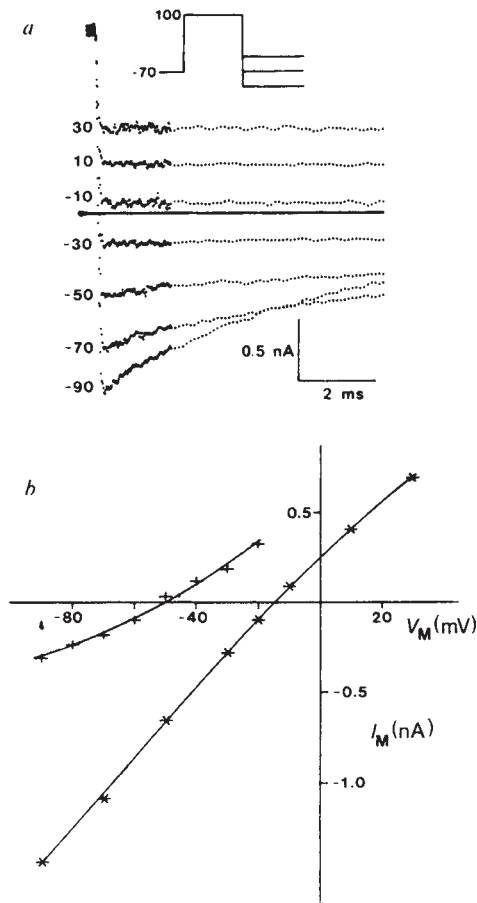


Fig. 2 K dependence of the reversal potential. The pulse protocol used to obtain the instantaneous current-voltage relationship is shown in the inset of *a*. The initial depolarization to +100 mV activated the outward current. The membrane was then stepped to various potentials and the resulting instantaneous change in current measured. For each trace shown in *a*, the first few points were taken before the pulse to provide a current baseline. Sampling was then stopped and restarted 200 μs before the second voltage step was applied. The voltage of the second step is indicated alongside each current trace. Pipettes were filled with the same solution as for Fig. 1 and the external solution had the following composition (mM): 85 NaCl, 60 KCl, 5 CaCl₂, 2 MgCl₂ and 10 HEPES. The magnitudes of the instantaneous currents shown in *a* (which were measured ~150 μs after the step because of the limited frequency response) are plotted in *b* (*); it can be seen that the current reverses direction at ~-15 mV. When the external solution contained only 15 mM K⁺ (+) the reversal potential shifted to ~-50 mV. Cells were attached with OKT11 and had been stimulated with succinyl concanavalin A for 20 h.

suspension (Table 1), again indicating selective adhesion. This adhesion method is accomplished by a simple protocol, is highly reproducible and could therefore be useful for a wide range of electrophysiological studies of specific cell types. In general, wherever cell types are distinguishable by their surface antigens, this method could be used to isolate particular cell types from heterogeneous populations.

We used this method to attach human lymphocytes for our patch clamp experiments. Cells were adhered with monoclonal antibody OKT3, 4 or 11. OKT11 is used for identification of human peripheral T lymphocytes; it binds to >95% of the cells that are E rosette-positive. The advantage of using OKT11 rather than OKT3 is that it is not mitogenic, and its use ensures that unstimulated cells are not activated by exposure to mitogenic antibody. We studied primarily OKT11-adhered cells, and in future studies we shall investigate those T-cell subsets predominantly recognized by OKT3, OKT4, OKT8 or other antibodies.

Table 1 Specificity of cell adhesion with monoclonal antibodies

Monoclonal antibody	% Positive in suspension	% Positive* bound to dish (direct immunofluorescence)	% Positive† bound to dish (indirect immunofluorescence)
OKT3	75.5	84	100
	74.9	85	
OKT4	49.9	64	100
OKT8	28.9	58	
OKT11			100

Human T lymphocytes were prepared from heparinized human venous blood which was collected from healthy donors, separated by gradient centrifugation using a modified Ficoll-Hypaque technique²⁵, suspended in minimal essential medium (MEM) or Hank's (Ca-Mg-containing medium) with 15% mixed human AB serum and incubated at 50×10^6 cells per flask (20 ml) in T-150 tissue culture flasks for 1.5 h. The supernatant was gently decanted, centrifuged at 1,500 r.p.m. for 10 min, resuspended in MEM and cultured in a 5% CO₂ incubator at 37 °C as described previously²⁵. Goat anti-mouse IgG (Tago Inc., Burlingame, California; 1.5 ml of a 1:10 dilution in Tris buffer, pH 9.5) was added to a Falcon Petri dish (bacterial grade, 13 × 100 mm) at 0 °C and incubated for 40 min. After this time, the dishes were rinsed with cold Tris buffer then with cold phosphate-buffered saline (PBS, pH 7.2) and cells were poured onto the plate and placed in the cold (4 °C) for ≥ 2 h. Coverslips (Corning) were treated in exactly the same way as the Falcon Petri dishes. Cells (1×10^6 ml⁻¹) suspended in 1 ml PBS/10% fetal calf serum (FCS) were incubated with fluorescein-labelled OKT3, 4 or 8 (0.5, 0.5 and 0.25 µg per sample, respectively) for 30 min, centrifuged and resuspended in 0.5 ml PBS. 0.25 ml was added to each coverslip and allowed to stand for 30 min at 0 °C. The coverslips were then washed in three successive rinse wells containing PBS and counted using a Zeiss fluorescence microscope (×40). In addition, 0.25-ml samples from the cell suspension were assayed using an Ortho-Spectrum III flow cytometer. Indirect immunofluorescence assays were carried out as described above, except that unlabelled OKT3, OKT4 and OKT11 (1 µg per 10^6 cells) were used. After incubation of the cell suspension on the coverslip for 30 min at 0 °C, the supernatant was removed and 100–200 µl of fluorescein-Tago (1:20 dilution) were added. The coverslips were then washed and the cells counted. All suspensions contained <1% monocytes. Those cells incubated with no antibody showed only weak autofluorescence.

* Fraction of labelled cells in 100–200 cells observed.

† No non-fluorescent cells were attached.

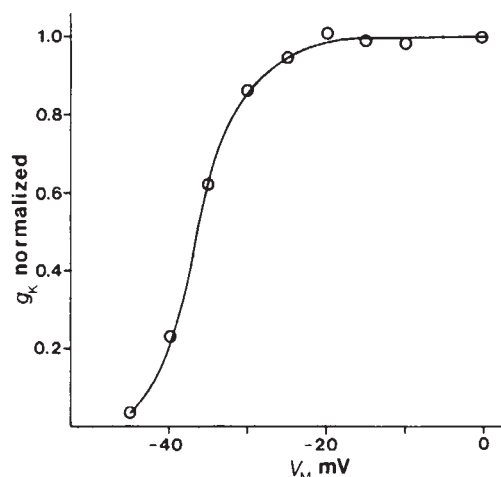


Fig. 3 Relationship of conductance to voltage. As the instantaneous I - V curve with 60 mM external K⁺ is approximately linear, its slope was used as a measure of the K conductance activated during the initial depolarization (see also the pulse protocol in Fig. 2a). The activating voltage was varied in the range of membrane potentials indicated (V_M). The data points represent the average values obtained for three different cells 10–15 min after breaking into the cell. The midpoints of the individual conductance-voltage curves were at -35, -36 and -38 mV, indicating that they were in approximately the same position. The external solution contained (in mM): 85 NaCl, 60 KCl, 5 CaCl₂, 2 MgCl₂ and 10 HEPES (pH 7.3) and the internal solution contained (in mM): 100 KCl, 40 KF, 2 MgCl₂ and 10 HEPES (pH 7.3). Fluoride was included in the pipette solution because it increased the recording time in each cell. The K currents and the conductance-voltage relationship were not significantly different in the presence or absence of fluoride. The cells had been stimulated with succinyl concanavalin A for 24 h. Series resistance compensation was used as described previously²⁷.

Macroscopic currents were recorded from human T lymphocytes using the whole-cell recording configuration of the patch clamp technique¹². Gigaohm seals were routinely obtained on these cells with 2–5 MΩ borosilicate electrodes. When lymphocytes are depolarized, outward currents are generated (Fig. 1). Both the magnitude of the current and its activation kinetics increase as the membrane potential is made more positive. As a result of prolonged depolarization, the outward current inactivates (see Fig. 1b). Inactivation is manifested both as a decline in current during depolarization, and as a decrease in current magnitude during a train of voltage clamp steps.

Several lines of evidence indicate that K channels carry the outward current illustrated in Fig. 1. For example, we have shown that the reversal potential for the current is close to the K equilibrium potential. Reversal potentials were determined from instantaneous current-voltage relationships (see Fig. 2). With 60 mM K⁺ outside and 140 mM K⁺ inside, the current reversed at ~-15 mV whereas the K equilibrium potential predicted from the Nernst equation is -21 mV. When the external solution contained 15 mM K⁺, the reversal potential shifted to -50 mV; the predicted shift is to -56 mV. Thus, the channel appears to be K-selective, but we have not conclusively identified the type(s) of K channels present. Agents that block K channels in other preparations block this conductance also. We have found that quinine (100 µM) reversibly blocks outward currents in human lymphocytes, which is consistent with previous reports that both the volume-induced and A23187-induced K and ⁸⁶Rb effluxes in these cells are blocked by quinine¹⁵. Cs⁺ blocks K channels in several preparations^{16,17}, and we also find that the K currents in human T cells are abolished when K⁺ in the patch electrode is replaced with Cs⁺.

We were interested in comparing the K currents in mitogen-stimulated cells with those in unstimulated cells, because there are several precedents for mitogen-induced increases in plasma

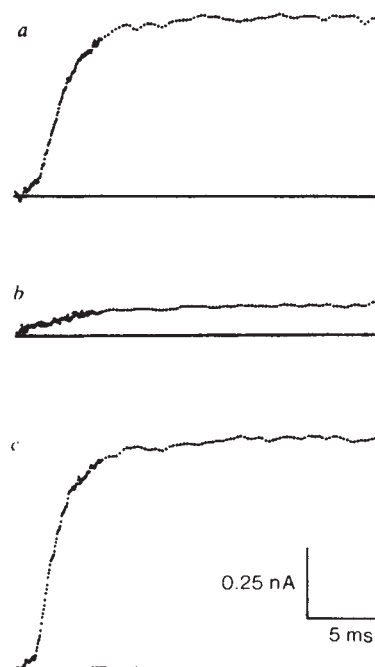


Fig. 4 External Ni²⁺ reduces K⁺ currents in lymphocytes. The currents shown were generated by pulses to +50 mV and represent typical examples under the following conditions. a, Control current recorded in a solution of the following composition (in mM): 138 NaCl, 5 CaCl₂, 2 MgCl₂, 5 KCl and 10 HEPES (pH 7.3); b, after addition of 1 mM Ni²⁺ to the external medium; c, after returning to the control solution. Ni²⁺ clearly reduces the magnitude of the K current and the effect is reversible. The pipette solution was the same as for Fig. 3. Cells were stimulated with succinyl concanavalin A for 24 h.

membrane ion permeabilities¹⁸⁻²⁰. Lymphocytes which had been stimulated with 50 $\mu\text{g ml}^{-1}$ succinyl concanavalin A in culture for 20 h showed a 1.9-fold increase in K current. The current magnitude at +100 mV averaged 0.67 ± 0.31 nA (mean $\pm$ s.d.) in 15 unstimulated cells and 1.27 ± 0.66 nA in 21 stimulated cells, a difference that is significant ($P < 0.01$). It is not yet clear exactly what the relationship is between stimulated growth and increased K conductance. Additional studies are under way to determine whether the increase in conductance is due to an increase in number of channels or an increase in single-channel conductance.

To further characterize the K channels in mitogen-stimulated cells, we studied the relationship between membrane voltage and K conductance (g_K). Figure 3 shows that the threshold for g_K activation in human lymphocytes is ~ -45 mV, the midpoint of the curve is -36 mV and the conductance saturates at -20 mV. The maximum g_K averaged 11.8 ± 2.8 nS (mean $\pm$ s.d.) in 9 cells.

There is indirect evidence that the K conductance in human lymphocytes may, in part, be due to the presence of calcium-activated K channels. For example, in the presence of extracellular calcium, the Ca^{2+} ionophore A23187 causes a severalfold increase in ^{86}Rb efflux and uptake¹⁵. In addition, Tsien *et al.*²¹ reported that in stimulated mouse thymocytes a rise in the concentration of intracellular free Ca was accompanied by cell hyperpolarization.

Therefore, we tried to obtain further evidence for the existence of Ca-activated K channels in human lymphocytes. One approach involved the use of Ni^{2+} , which is known to block Ca channels in other preparations²². Addition of 1 mM Ni^{2+} to the external medium reversibly reduced the K current, as illustrated in Fig. 4. This could reflect decreased Ca^{2+} entry which would result in a decreased activation of Ca-activated K channels. Alternatively, Ni^{2+} could be directly blocking the K channels, although we are not aware of any reports of this effect. Using Cs^+ -filled electrodes to block the K conductance, we have been unable to observe any macroscopic Ca currents in these cells. Calcium channels may well be present in PBLs, but in numbers too small to detect as macroscopic current. By contrast, it is interesting that Fukushima and Hagiwara²³ found Ca channels in cultured myeloma cells; perhaps transformation is accompanied by an increased number of Ca channels²⁴.

In summary we have patch-clamped human T lymphocytes and identified potassium channels in both resting and mitogen-stimulated cells. Selective adhesion with monoclonal antibodies should allow us to characterize the membrane conductances of individual subsets of lymphocytes in a variety of conditions in which cellular and humoral immune responses occur.

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Retinoblastoma—origin from a primitive neuroectodermal cell?

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The histogenesis of retinoblastoma, the most common intraocular neoplasm of childhood¹, remains controversial. Previous studies have attributed the origin of the tumour to neuronal, glial or primitive stem cells of retina²⁻⁷. In the study described here we have used immunofluorescence to search for the presence of a neuronal marker, neurone-specific enolase (NSE)^{8,9} and a glial marker, glial fibrillary acidic protein (GFAP)^{10,11}, in the cells of the human retinoblastoma line Y-79 (ref. 4), before and after successful differentiation into neuronal and glial-like cells. We found that all undifferentiated cells contain both NSE and GFAP, whereas the differentiating neuronal and glial-like cells gradually lose one marker and selectively express the marker that correlates with their morphology. Our results support the notion that retinoblastoma originates from a primitive bipotential (or multipotential) neuroectodermal cell.

Monolayers of Y-79 cells were obtained in dishes precoated with poly-D-lysine and fibronectin and kept in both serum-supplemented and serum-free defined media¹². $\text{N}^6, \text{O}^{12}$ -dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP) was added to some dishes with serum-supplemented media¹². Cells growing in serum-supplemented medium retained their conventional morphology of small round cells¹² (Fig. 1a), whereas cultures growing in serum-free defined medium showed extensive neuronal differentiation, consisting of a gradual increase in cells with neuritic-type processes (up to 30%) and rosette formation (Fig. 1b). In contrast, dbcAMP caused a small but significant increase in flat, glial-like cells (up to 10%) (Fig. 1c).

Indirect immunofluorescence was done in order to investigate the presence of NSE and GFAP in these cells. Normal rabbit serum (1:50) was always used as a negative control. A human neuroblastoma line, SMS-SAN (ref. 13) and a human glioma line, U-251MG (ref. 14), were used as positive and negative controls for each marker; human normal fibroblasts maintained in culture for >1 yr were used as a negative control for both markers. The specificities of the antisera were further tested by primary tissue staining of 5- μm sections from paraffin blocks of formalin-fixed tissue, by using the immunoperoxidase (PAP) method of Sternberger *et al.*¹⁵. The tissues examined included both positive and negative controls: normal human brain and spinal cord (tested with both antisera); an olfactory neuroblastoma, a pineoblastoma, a medulloblastoma, a mixed ependymoma-oligodendroglioma, a spinal and a cerebral astrocytoma, and a mixed grade 4 astrocytoma-meningeal sarcoma (tested with anti-GFAP); and five neuroblastomas, five lymphomas, one medulloblastoma, five Ewing's sarcomas and four soft tissue sarcomas (tested with anti-NSE). The diagnoses for all the above cases had been documented by light microscopy and when necessary, by electron microscopy and by additional means such as study of surface markers for lymphomas.

Fig. 1 Monolayers of the Y-79 cell line. *a*, Undifferentiated cells at 6 days in culture. Cells were seeded in 35-mm plastic dishes which had been pretreated with poly-D-lysine (0.2 mg ml^{-1}) at 23°C for 6 min, washed once with minimum essential medium (MEM) containing 2 mM glutamine and 100 U ml^{-1} penicillin, and coated with fibronectin ($5 \mu\text{g ml}^{-1}$) at 37°C for 30 min. The culture medium consisted of the medium described above, supplemented with 10% fetal bovine serum ($\times 190$). *b*, Cells demonstrating neuronal differentiation, at 22 days in serum-free, defined medium.

Cells were seeded as for *a*, incubated for 2 h in serum-containing medium, and washed once with MEM. Finally, serum-free medium was added (supplemented with $5 \mu\text{g ml}^{-1}$ insulin, $10 \mu\text{g ml}^{-1}$ transferrin, 6.3 ng ml^{-1} progesterone, 8.8 ng ml^{-1} putrescine and 4 ng ml^{-1} sodium selenite) ($\times 125$). *c*, Flat cells with short cytoplasmic projections, resembling astrocytes (22 days in culture). In the centre there is a clump of small, closely packed undifferentiated cells. These cells were cultured in serum-containing medium; dibutyryl cyclic AMP (4 mM) was added at 3-day intervals beginning on day 8 of culture (five doses in total) ($\times 155$).

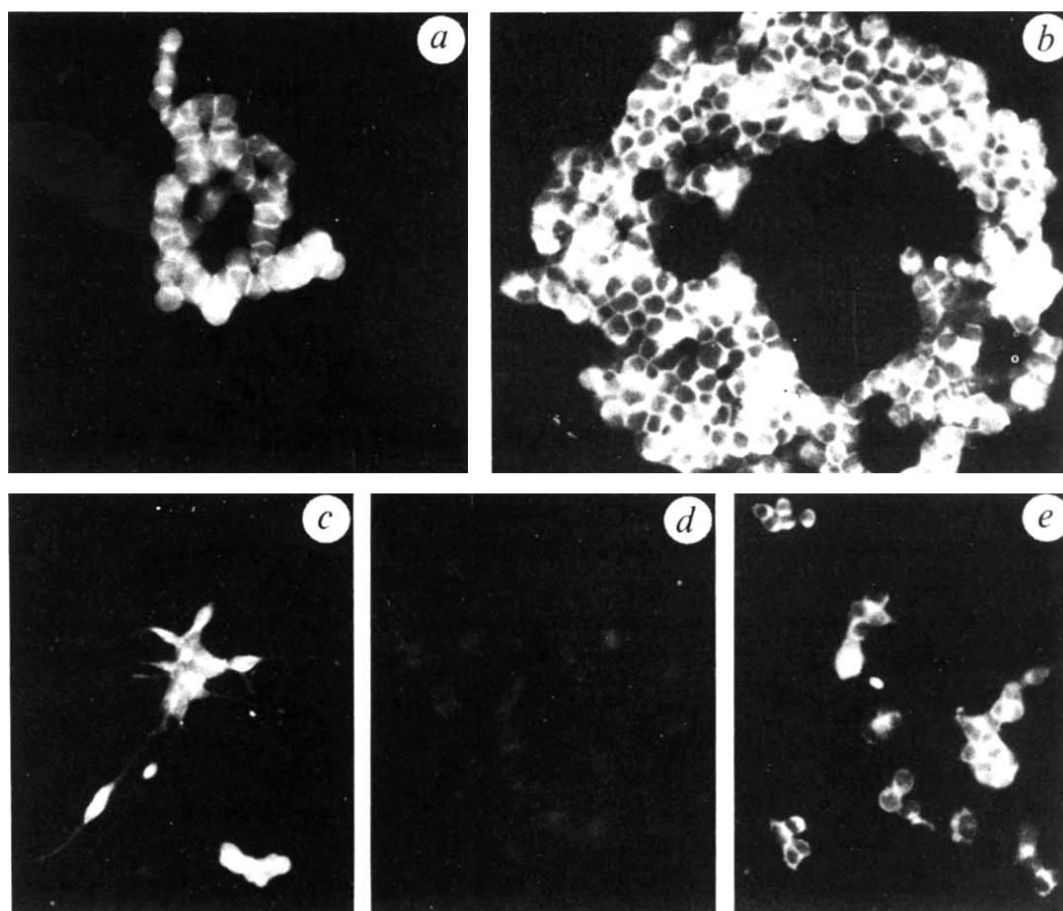
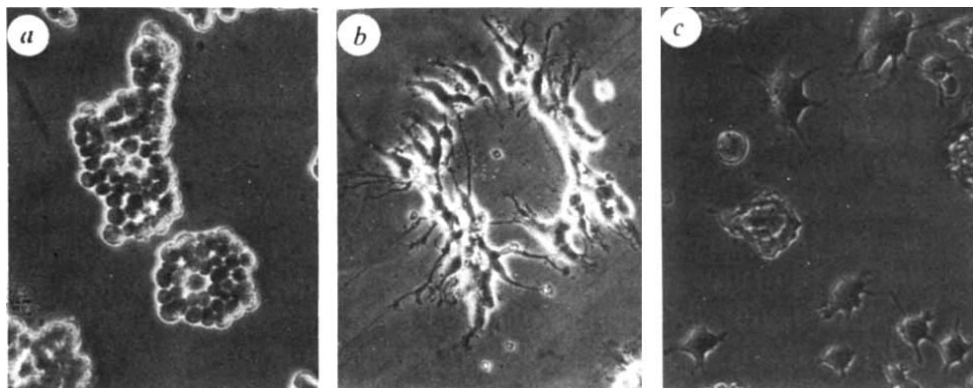


Fig. 2 Indirect immunofluorescence staining of Y-79 cells. Cells were fixed and incubated with anti-serum as described in Table 1. *a*, Undifferentiated round cells positive for NSE ($\times 257$). *b*, Undifferentiated round cells positive for GFAP ($\times 185$). *c*, Cells with neuronal differentiation positive for NSE ($\times 135$). *d*, Flat glial-like cells negative for NSE. The flat cells are isolated; they are growing without forming clumps, chains or rosettes, in contrast to the undifferentiated or neuronal cells ($\times 150$). *e*, Flat cells positive for GFAP ($\times 135$).

The results of staining for the control and retinoblastoma cell lines before and after differentiation are given in Table 1. The pattern of staining for the control cell lines confirms the specificity of the antisera used in this study. Positive NSE staining was only observed in neuroblastoma cells. Anti-GFAP antiserum stained only glioma cells as reported previously¹⁴⁻¹⁶, thereby obviating the possibility of cross-reactivity between GFAP and tubulin. Normal fibroblasts remained repeatedly negative for either marker, providing indirect evidence that our antisera did not cross-react with vimentin. A highly specific staining pattern was observed with immunoperoxidase also: that is, the neurones of the sections from brain and spinal cord as well as the cross-sectioned neuritic axons in the white matter of the spinal cord and adjacent spinal nerves were positive for NSE and negative for GFAP. In contrast, astrocytes in both tissues stained intensely positive for GFAP and remained negative for NSE.

Among the other tissues examined, the olfactory neuroblastoma, pineoblastoma and the oligodendrocytic and sarcomatous components of the glial tumours, were negative for GFAP, whereas the astrocytomas and ependymoma were positive for GFAP, as reported previously¹¹. The neuroblastomas were positive for NSE, whereas the lymphomas, Ewing's sarcoma and all soft tissue sarcomas were negative for NSE, as expected^{17,18}. The medulloblastoma was generally negative for both NSE and GFAP, except for occasional GFAP-positive cells, previously interpreted as glial differentiation of the tumour¹⁹.

All cells of the Y-79 human retinoblastoma line showed the simultaneous presence of NSE and GFAP before their differentiation into neuronal and glial-like (flat) cells respectively (Fig. 2*a, b*). The anti-GFAP antiserum gave a higher intensity of staining overall than the anti-NSE antiserum. One-third of the cells with neuronal differentiation in the defined medium were

Table 1 NSE and GFAP reactivity of retinoblastoma and control cells *in vitro*

Type of cell line	NSE reactivity	GFAP reactivity
(1) Retinoblastoma (Y-79)		
Undifferentiated	+	+
Differentiated*		
a Neuronal	+	+/-
b Glial-like	-	+
(2) Neuroblastoma (SMS-SAN)	+	-
(3) Glioma (U-251-MG)	-	+
(4) Normal fibroblasts	-	-

All cultures were fixed in absolute ethanol at -200°C for 4 min and subsequently washed in phosphate-buffered saline (PBS). Primary antisera raised in rabbit against rat NSE (1:50; Polysciences, Warrington, Pennsylvania) and human GFAP (1:100; Accurate, Westbury, New York) were applied to all cultures at 4°C for 24 h. The cultures were subsequently washed in PBS, incubated for 1 h with swine anti-rabbit IgG linked to fluorescein isothiocyanate (FITC) (1:20), washed again in PBS and observed under a Zeiss Standard microscope equipped with an epifluorescence illuminator and FITC narrow-band filter.

* The staining results for NSE and GFAP refer only to the differentiated cells. The cells which remained undifferentiated always retained both markers. One-third of the cells with neuronal differentiation became GFAP-negative, whereas all glial cells became NSE-negative.

GFAP-negative. All neuronal cells showed a higher intensity of NSE reactivity (Fig. 2c) than the undifferentiated cells. On the other hand, flat cells in dbcAMP-treated cultures were always negative for NSE (Fig. 2d), whereas most of them were positive for GFAP (Fig. 2e).

The simultaneous presence of NSE and GFAP in the retinoblastoma cells suggests that these cells originated from a primitive neuroectodermal cell capable of differentiation into neuronal and glial elements. Thus far, NSE and GFAP have not been detected simultaneously in another tumour either *in vivo* or *in vitro*. Morphological transformation of the retinoblastoma cells into neuronal and glial-like cells with specific staining patterns for NSE (neuronal) and GFAP (glial) reinforces the concept of the primitive bipotential nature of retinoblastoma. The fact that two-thirds of the cells in serum-free medium, despite their morphological differentiation into neuronal cells, retained their GFAP positivity suggests that they were in an earlier stage of biochemical differentiation. Both neuronal and glial cells have been described previously in retinoblastomas *in vivo* and *in vitro*^{3,6,7,20}. However, the neoplastic nature of the glial cells in retinoblastoma has so far been a moot point. The present system obviates this problem, as it appears that the neoplastic cells differentiate into both neuronal and glial-like cell types and express biochemical patterns which correlate with the morphological phenotype of the differentiating cells.

The potential of the embryonal retinal cell to differentiate into neurones and glial cells has been shown by Ohnishi²¹ and Fujisawa *et al.*²² in dissociated neuroretinal cells of chick embryos. We have shown here that the same phenomenon can occur in the neoplastic counterpart of retinal cells—the retinoblastoma cell. An analogous situation exists in differentiating neuroblastoma (neural and Schwann cells)^{23,24} and medulloblastoma (neuronal and glial cells)¹⁹. In both cases, the primitive tumour cells are presumed to have differentiated into neuronal and supporting cells. One can speculate that retinoblastoma is the result of exuberant proliferation of residual primitive retinal cells, or that dedifferentiation of mature retinal cells towards a more primitive neuroectodermal cell is necessary as a prelude to the neoplastic behaviour. It is interesting that the coexistence of neurofilaments (previously seen only in neurones) and vimentin (previously seen in some forms of glia) has been reported recently in the axonless horizontal cell of the outer plexiform layer in mouse retina, leading to the conclusion that this cell might represent an intermediate stage of differentiation between neuronal and glial cells²⁵.

This is the first time that *in vitro* differentiation of retinoblastoma has been achieved and studied morphologically and cytochemically. We believe that the present study will contribute to our knowledge of the origin and biological behaviour of retinoblastoma. It may also provide a system to assess the effects of various agents on tumour maturation *in vitro*, which may ultimately effect a potential cure *in vivo*.

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A novel 6:10 chromosomal translocation in the murine plasmacytoma NS-1

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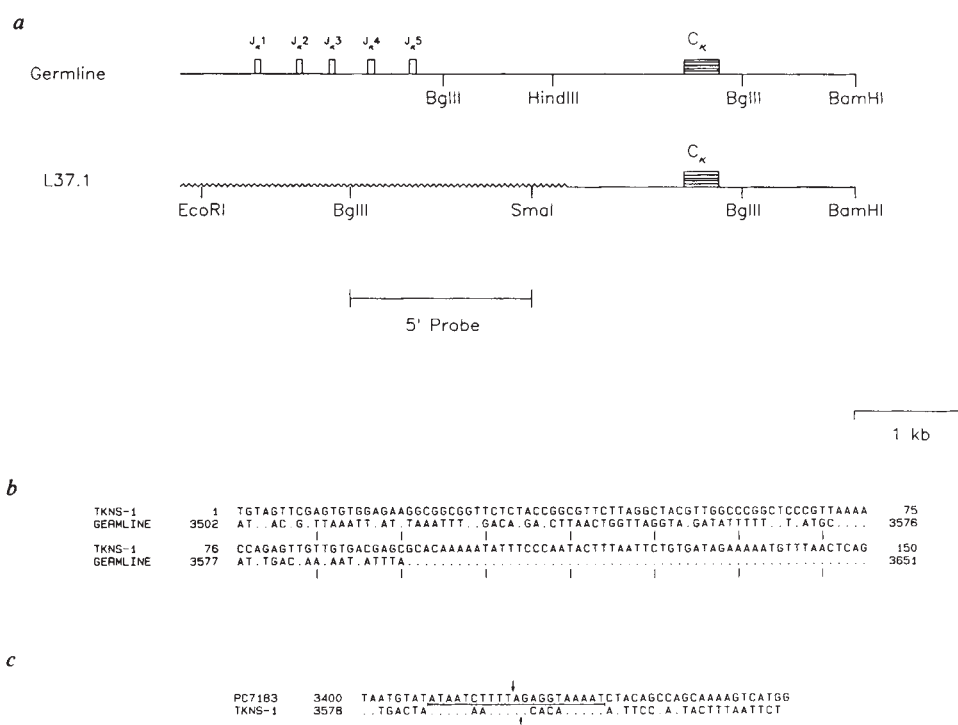
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Specific chromosomal abnormalities are regularly associated with many murine and human malignancies^{1,2}. In particular, the majority of murine plasmacytomas and human Burkitt's lymphomas contain a characteristic translocation which results in the juxtaposition of a cellular oncogene, *c-myc*, with the immunoglobulin heavy-chain gene locus^{3–7}, and this rearranged *c-myc* directs the synthesis of qualitatively and quantitatively abnormal transcripts which may have an aetiological role in the development of the transformed state in lymphoid malignancies^{8,9}. Similarly, rearrangement and abnormal expression of *c-myb* (ref. 10) and *c-mos* (ref. 11) has been reported in other murine lymphoid tumours. Here we describe a novel 6:10 chromosomal translocation in the murine plasmacytoma cell line NS-1 which juxtaposes the immunoglobulin *C_κ* gene with a single-copy sequence of unknown function. The NS-1 plasmacytoma is a frequently used fusion partner in hybridoma production¹² and is known to contain a rearranged *c-myc* gene^{4,13} and a genetic element which transforms normal mouse fibroblasts in DNA-mediated transfection assays¹⁴. We conclude that individual B-cell tumours may contain multiple chromosomal translocations perhaps relevant to oncogenesis.

Phage clones containing *C_κ* sequences were isolated from a genomic library of hybridoma DNA prepared using the L47.1

Fig. 1 a, An aberrantly rearranged C_{κ} gene. Genomic DNA was isolated from the HGAC9 hybridoma (secreting antibody to group A streptococcal carbohydrate³²) and partially digested with *Mbo*I to allow construction of a library in L47.1 phage (see ref. 15). On screening with a murine C_{κ} gene probe, several clones were obtained which contained a novel sequence juxtaposed to C_{κ} . Restriction enzyme maps for the germ-line C_{κ} locus and for one of the rearranged clones, L37.1, are shown. The rearranged sequence begins just 3' to the germ-line *Hind*III site and is denoted by a wavy line. A *Sma*I/*Bgl*II digest was used to generate a probe for this sequence (5' probe) which was subcloned into M13mp8 (BRL). **b**, DNA sequence at the site of the T_{κ} NS-1 translocation site. The T_{κ} NS-1 sequence was determined by the chain termination method of Sanger³³. The germ-line sequence is from ref. 34. Numbering of the T_{κ} NS-1 sequence is arbitrary; numbering of the germ-line sequence reflects ref. 34. Homologous bases are indicated with dots. The remainder of the T_{κ} NS-1 sequence extending towards the C_{κ} gene agrees precisely with the published sequence for this region³⁴. **c**, Two potential target sites for translocation in the C_{κ} intervening sequence. The points of translocation for PC7183²⁰ and for T_{κ} NS-1 (this paper) within the germ-line C_{κ} intervening sequence³⁴ are compared (arrows). The solid line denotes a 21-bp sequence, straddling the recombination points, which is closely homologous for both translocation sites.



phage vector¹⁵. Several overlapping clones were obtained which contained the C_{κ} gene and an aberrant sequence joined 1 kilobase (kb) 5' to the C_{κ} gene (Fig. 1a). A 1.6-kb fragment derived entirely from this novel 5' sequence was prepared by digestion with *Sma*I and *Bgl*II endonucleases (Fig. 1a) and used to probe Southern blots of BALB/c sperm DNA and DNA from various cell lines. Figure 2 shows that this probe identifies a single-copy sequence in germ-line (sperm) DNA but that both the hybridoma cell line from which the library was prepared (HGAC9) and the parent line NS-1 contain a rearranged copy of this sequence which results from the juxtaposition of C_{κ} (Fig. 1a). Thus, the C_{κ} rearrangement which we cloned from a specific hybridoma was present in the common parent cell line.

To identify the chromosomal location of the single-copy sequence aberrantly joined to the C_{κ} gene in NS-1 cells, we used mouse-hamster somatic cell fusion lines which retain limited numbers of different murine chromosomes¹⁶. Southern blot analysis of DNA from a number of such lines using our 1.6-kb probe unambiguously localized this sequence to chromosome 10 (Fig. 3). Thus, a 6:10 translocation in the NS-1 plasmacytoma fuses the C_{κ} gene on chromosome 6 to a single copy element from chromosome 10. We call this element T_{κ} NS-1.

Because of the recent demonstration of translocations involving *c-myc* and immunoglobulin genes in murine plasmacytomas, we sought to identify a retroviral oncogene within the T_{κ} NS-1 sequence. Using cloned *v-onc* probes, we were unable to identify any sequence homologous to *src*, *myb*, *myc*, *abl*, *ras*^H, *ras*^K, *fes*, *yes*, *sis*, *mos*, *rel*, *bas*, *erb*, *fms* or *fos* in the ~8 kb of DNA 5' to C_{κ} in this cloned translocation (refs 17, 18 and data not shown). Similarly, the T_{κ} NS-1 probe did not detect any rearranged sequences in NIH3T3 fibroblast transformants generated using murine plasmacytoma DNA which therefore contain the previously described plasmacytoma transforming element known to be present in NS-1 cells (ref. 14 and R.M.P. and M.A. Lane, unpublished observation). We conclude that T_{κ} NS-1 probably does not include a previously described oncogene sequence.

As 90% of BALB/c plasmacytomas appear to contain a 12:15 chromosomal translocation^{3-5,9,19}, we examined 10

different B-cell tumours using Southern blotting and the T_{κ} NS-1 probe to assess the frequency of this specific rearrangement. No other examples of this translocation event were identified. In this sense the T_{κ} NS-1 6:10 translocation resembles a complex 6:12:15 translocation recently characterized by Van Ness *et al.*²⁰ which was also localized to the C_{κ} intervening sequence and which is also exceedingly infrequent in murine plasmacytomas (M. Weigert, personal communication). Thus, if these translocations predispose to malignant transformation, they are clearly not essential for the development of B-cell neoplasia. In this regard, we may speculate that the C_{κ} intervening sequence could be particularly susceptible to chromosomal rearrangements.

DNA sequence analysis of T_{κ} NS-1 at the translocation break-point reveals little homology between the C_{κ} intervening sequence and the novel element derived from chromosome 10 (Fig. 1b). Involvement of immunoglobulin switch sequences in the aberrant rearrangement of *c-myc* has suggested that normal B-cell differentiation mechanism may be involved in the genesis of the 12:15 translocation in plasmacytomas⁴⁻⁶. This explanation cannot apply to either the T_{κ} NS-1 translocation or the 6:12:15 translocation described in PC7183²⁰ because both occur in a region of the C_{κ} intervening sequence which does not ordinarily undergo DNA rearrangements.

Interestingly, careful comparison of the translocation points within the C_{κ} intervening sequence found in NS-1 and PC7183, which are separated by 180 bases, reveals a region of homology (15/21 base pairs, bp) straddling these sites (Fig. 1c). A search of the entire C_{κ} locus sequence, nearly 6 kb, identified these as the only two regions containing a sequence homologous to the T_{κ} NS-1 target site. This raises the possibility that some translocations occur via a site-specific mechanism.

Three properties distinguish the region immediately 5' to the C_{κ} gene. First, heteroduplex analysis has established an area of conserved sequence between human and mouse DNA about 700 bp 5' to the C_{κ} gene²¹. In addition, this region contains a DNase-I hypersensitive site, felt to reflect gene activation for increased expression²², in approximately the same position as the man-mouse sequence homology²³⁻²⁵. Finally, the C_{κ} inter-

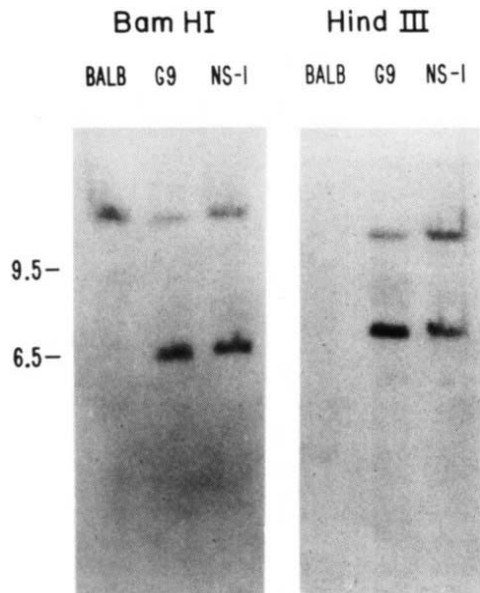


Fig. 2 The L37.1 rearranged C_{κ} gene is derived from the NS-1 hybridoma parent. Southern blots of 10 μ g genomic DNA from BALB/c sperm, HGAC9 hybridoma cells or NS-1 plasmacytoma cells digested with *Bam*HI (left panel) or *Hind*III (right panel) were performed using the 1.6-kb *Sma*I/*Bgl*II 5' probe shown in Fig. 1 labelled to 10^8 d.p.m. with 32 P-nucleotide triphosphates by primer extension³⁵. After electrophoresis in 0.7% agarose, fragments were blotted onto nitrocellulose and hybridization was performed in 50% formamide at 42 °C as described previously³⁶. The blots were washed in 0.015 M sodium citrate, 0.15 M sodium chloride, 0.1% SDS at 68 °C for 2 h before autoradiography overnight at -70 °C using Kodak XAR-5 film (Eastman Kodak) and an intensifying screen. The positions of standard size markers derived from *Hind*III fragments of phage λ DNA are noted in kb at the left.

vening sequence has been shown to contain an enhancer element located 3' to the *Hind*III site which dramatically increases transcription of C_{κ} -containing plasmids introduced into murine lymphoid cells²⁶⁻²⁸. Although translocation may occur randomly throughout the genome, as has been proposed for retroviral transduction¹⁷, we find it provocative that two independent translocation events in murine plasmacytomas occur at highly homologous sites 180 bp apart in a sequence apparently organized to support active gene expression.

The NS-1 plasmacytoma line was developed from a mineral oil-induced tumour, MOPC-21, after adaptation to culture conditions and selection for sensitivity to hypoxanthine-aminopterin-thymidine medium¹² and we cannot be certain at which point during the genesis of NS-1 the T_{κ} NS-1 6:10 translocation occurred. The fact that recent isolates of MOPC-21 do not contain this translocation²⁹ may reflect deletion of a κ allele during propagation of this tumour. In addition to the T_{κ} NS-1 translocation, the NS-1 cell line contains a characteristic 12:15 translocation event of murine plasmacytomas, an activated oncogene common to most murine plasmacytomas¹⁴, and a rearrangement in *c-mos* apparently analogous to that described in XRPC24 (ref. 11 and N. Takahashi, personal communication). Although the simultaneous coexistence of these four sequence abnormalities may in part reflect chromosomal instability within cultured cell lines, it is significant that three of these four abnormalities involve genes having known transforming potential. One possible explanation might be that these oncogenes act in concert, together providing growth advantages not present when only one is expressed³⁰ or perhaps developed sequentially, reflecting multiple stages in oncogenesis^{14,31}. Thus, chromosomal translocations in neoplastic cells may provide a window for the selective identification of important genetic elements involved in differentiation and growth control.

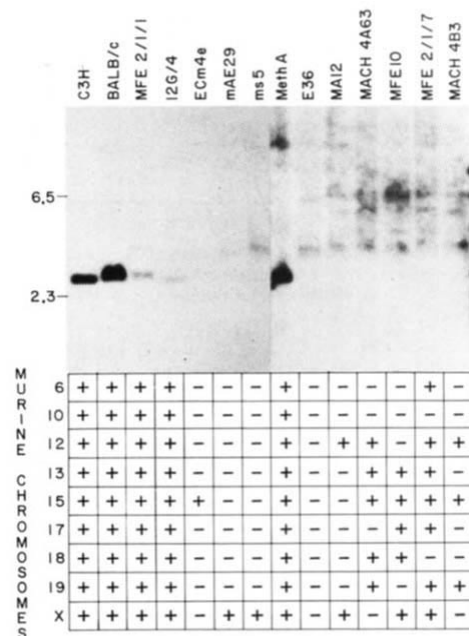


Fig. 3 Chromosomal location of T_{κ} NS-1. Patterns obtained when genomic DNA from the indicated mouse strains (C3H, BALB/c), from a BALB/c sarcoma (MethA), a Chinese hamster cell line (E36) and mouse-hamster somatic cell fusion lines generated using MethA as a parent (ms5, mAE29, MA12) or BALB/c embryo fibroblasts as parent cells (MFE2/1/7, MFE2/1/1, MFE10), or A/HeJ macrophages as parent cells (MACH4A63, MACH4B3) is analysed by Southern blotting as described in Fig. 2 legend. The origin of cell line Ecm4e has been described previously³⁷. The DNA was digested with *Eco*RI which produces a 2.8-kb band that hybridizes with the *Sma*I/*Bgl*II probe used in Fig. 2. The specific murine chromosomes retained in each hybrid line are indicated with a plus sign. To simplify the figure, the complete karyotype of each cell line is not indicated. Chromosome 12 has been rearranged in the MFE10 cell lines. Hazy bands larger than 3.0 kb reflect cross-hybridization with homologous hamster-derived sequences. The positions of standard size markers are again indicated in kb at the left. Additional hybrid cell lines examined included MFE8, which contains all murine chromosomes except 6 and Y and which was positive for T_{κ} NS-1 as was 12G/3, which lacks murine chromosomes 1, 3, 4, 7, 9, 11 and Y. Cell line 12G/2 lacking chromosomes 1, 2, 3, 4, 7, 9, 10, 11, 12, 14, 18, 19 and Y also lacked the T_{κ} NS-1 band. Chromosomes not shown in the figure but present in the cell lines were as follows: MFE2/1/1: 1, 2, 3, 4, 7, 8, 9; 12G4: 5, 14, 16; Ecm4e: 14; mAE29: 14; MACH4A63: 2, 4, 7, 8, 16; MFE10: 1, 2, 7, 8, 9, 11, 14, 16; MFE2/1/7: 1, 2, 3, 7, 8, 9; MACH4B3: 2, 7, 16.

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Activation of N-ras gene in bone marrow cells from a patient with acute myeloblastic leukaemia

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Human tumour cell lines of various histological origin contain genes that can transform NIH 3T3 cells in culture^{1,2}. Most frequently the gene is an activated K-ras gene, more rarely an activated H-ras gene, and sometimes the recently discovered N-ras³⁻¹¹. Other transforming genes, distinct from ras, have been found in B- and T-cell leukaemias^{9,12}. Since most of the transforming genes have been identified in cell lines, it is still unclear at what stage the genes become activated. We have therefore initiated a study to determine if the presence of a transforming gene correlates with the clinical course of a malignant disease. Here we demonstrate the presence of a transforming N-ras gene in bone marrow cells from a patient with acute myeloblastic leukaemia at the outbreak of the acute disease phase. Fibroblast DNA from the same patient was not transforming. In contrast to HL-60 cells^{13,14}, no alteration of the myc gene was detected.

A 9-year-old girl with anaemia and leukocytosis was admitted to the hospital where an acute myeloblastic leukaemia (AML) M2 (ref. 15) was diagnosed. Clinical and laboratory findings are summarized in Table 1. An aliquot of this patient's diagnostic bone marrow aspirate, obtained before chemotherapy administration, was examined for the possible presence of a transforming gene. AML DNA was found to contain focus-forming activity in three consecutive experiments (Table 1a). Four foci from three different plates were picked using cloning cylinders and grown into cell lines of clearly transformed morphology. The DNA from these lines was prepared and tested in a secondary transfection experiment to determine whether the putative transforming gene could be transferred. Table 1b shows that this DNA could induce foci on NIH 3T3 cells.

DNA derived from primary and secondary foci was subjected to Southern blot analysis using BLUR-8¹⁶ as a probe for the detection of human repetitive Alu sequences in the transfection foci. Figure 1 shows that a representative primary focus (lane b) contains a large number of human repetitive DNA sequences. Three secondary foci (lanes c-e) share an Alu-containing fragment of ~8.8 kilobases (kb). A fourth secondary focus lacks

Table 1 Primary and secondary transfection experiments

a Primary transfection		
Source of DNA	Foci per no. of plates	Foci per µg DNA
Calf thymus	1/6	0.004
AML bone marrow	42/20	0.056
b Secondary transfection		
Primary foci		
T82/73-9A1	22/6	0.100
T82/73-10/2	12/6	0.053
T82/73-11/1	37/4	0.247
T82/73-10/3	22/6	0.100

The patient under study was in good general condition; the only abnormal findings were a moderate hepato-splenomegaly, slightly enlarged lymph nodes and a small perirectal abscess. The white blood cell count was $62 \times 10^9 \text{ l}^{-1}$ (normal range $5-12 \times 10^9 \text{ l}^{-1}$); most cells were atypical myeloblasts. There was thrombocytopenia with a platelet count of $55 \times 10^9 \text{ l}^{-1}$ ($150-400 \times 10^9 \text{ l}^{-1}$) and an anaemia with 91 g l^{-1} of haemoglobin ($120-140 \text{ g l}^{-1}$). The bone marrow was hypercellular with 68% atypical myeloblasts cytochemically positive for peroxidase and Sudan black, negative for Pas and nonspecific esterase. This, together with morphological criteria, established the diagnosis of acute myeloblastic leukaemia (AML), type M2 according to the FAB (French-American-British) classification¹⁵. No abnormal findings were detected by karyotype and chromosome banding analysis (H. J. Müller, data not shown). Bone marrow cells prepared by standard Ficoll centrifugation were lysed using 0.5% SDS in 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, and digested with proteinase K. DNA was prepared by phenol and chloroform-isoamyl alcohol extractions followed by ethanol precipitation and subsequently resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. NIH 3T3 cells, seeded at 5×10^5 cells per 10-cm dish 1 day before the experiment, were transfected with 37.5 µg DNA as described¹⁸. Medium was changed 6 h later, then twice weekly. Foci of morphologically transformed cells were counted after 14 days.

this fragment, but contains two fragments of ~20 and 6 kb. We tentatively conclude that the transforming activity is associated with the 8.8-kb fragment, and perhaps with the 20- or 6-kb fragment. To characterize the transforming gene further we tested its sensitivity to seven restriction enzymes by digesting primary focus DNA before the transfection experiment. Focus formation was reduced 10-fold or more by digestion with *EcoRI*, *HindIII*, *BamHI* or *PvuII*, while *XhoI*, *Sall* and *ClaI* did not reduce the focus-forming activity of the DNA preparation (data not shown). This restriction enzyme sensitivity pattern differs from the published patterns obtained from B- and T-cell leukaemias⁹, but is identical to a pattern obtained with a transforming gene present in a fibrosarcoma and also in an embryonal rhabdomyosarcoma line¹⁷. In a subsequent study⁸ by the same group this sarcoma-derived gene was cloned and identified as the N-ras gene.

Southern blot analysis was then used to determine whether the AML-derived transfection foci contained the N-ras gene as suggested by the restriction enzyme sensitivity experiment. The probe used was an *EcoRI*-*SstI* subfragment from a cloned 8.8-kb *EcoRI* fragment⁸ containing the 5' region of the N-ras gene given by Alan Hall. With this probe, we detected an 8.8-kb *EcoRI* fragment in DNA from a primary focus (Fig. 2b) and from three secondary foci (Fig. 2c-e). In a fourth secondary focus (Fig. 2f), a hybridizing fragment in the 20-kb region, corresponding to the upper Alu-containing fragment in Fig. 1f, was detected. The slower mobility of this fragment is probably due to loss of one of the *EcoRI* sites flanking the 8.8-kb fragment brought about by DNA shearing required by the transfection protocol¹⁸. We conclude that the transforming gene present in AML bone marrow represents the N-ras gene, part of which can be detected in most transfection foci as an 8.8-kb *EcoRI* fragment. Consistent with this interpretation are the results of immunoprecipitation experiments by H. P. Senn using a monoclonal anti-p21 antibody which show that secondary foci contain elevated levels of a ras protein (data not shown).

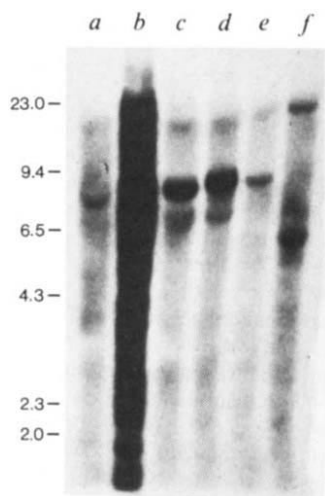


Fig. 1 Presence of human repetitive DNA in primary and secondary transfection foci. Aliquots (15 μ g) of cellular DNA were digested overnight with *Eco*RI, phenol-extracted, ethanol-precipitated and subjected to agarose (0.8%) gel electrophoresis. After transfer of DNA to nitrocellulose, hybridization was done in the presence of 10% dextran sulphate at 67 °C using 2×10^7 c.p.m. of BLUR-8 probe¹⁶ nick-translated to a specific activity of 1.4×10^8 c.p.m. per μ g DNA. The final wash of the filter was in $0.5 \times \text{SSC}/0.1\%$ SDS for 20 min at 65 °C. Autoradiography was at -70 °C for 16 h using Kodak X-Omat SO-282 and an intensifying screen. Cellular DNA was from: a, NIH 3T3 cells; b, primary focus T82/73-9A1; c, secondary focus T83/76-7b; d, secondary focus T83/76-9a; e, secondary focus T83/80-8; f, secondary focus T83/76-11a.

A transforming *N-ras* gene has been reported to be present in a neuroblastoma⁷ line, two sarcoma^{8,17} lines, the promyelocytic leukaemia¹¹ line HL-60, and, as presented here, in AML bone marrow cells at the outbreak of the acute phase of the disease. Thus, this gene is associated with tumours of different origins, but also with different maturation stages of leukaemias within the same lineage, namely the more primitive AML and the more differentiated promyelocytic leukaemia.

The HL-60 line has been shown to have an alteration of a second oncogene—an amplification of the *myc* gene^{13,14} which is associated with chromosome translocations in human B-lymphomas¹⁹⁻²². While it is assumed but not known whether the *N-ras* gene was already present as a transforming variant in the original HL-60 tumour biopsy, it has been shown that the *myc* amplification had occurred *in vivo*¹⁴. To determine whether we could detect a *myc* gene alteration in AML DNA, we examined DNA from the original bone marrow aspirate and DNA from skin fibroblasts established in culture from the same patient. Normal human spleen DNA from an unrelated donor and DNA from HL-60 cells were included in the analysis as controls. As a probe we used a *Cl*aI-*Eco*RI fragment of the human *myc* gene containing the 3' exon given by R. C. Gallo. We detected neither amplification nor altered mobility of the *myc* gene in our AML patient, while HL-60 DNA showed the expected^{13,14} amplification (Fig. 3). While we cannot exclude a minor *myc* gene alteration, it seems that *N-ras* activation in AML can occur independently of *myc* amplification/rearrangement. If indeed both the *N-ras* and *myc* genes have a role in the natural history of some myeloid leukaemias of which HL-60 may be a prototype, it would seem that in some cases, such as the more primitive AML, the activated *N-ras* gene exerts its effects independently of *myc*, and thus may require alterations of other genes.

To approach the question of whether the activation of the *N-ras* gene observed in this patient was a somatic event, we intend to compare the cloned activated gene with its alleles from the patient's fibroblasts. In three preliminary transfection experiments, this fibroblast DNA displayed a focus-forming activity of <0.005 foci per μ g DNA, which corresponds to

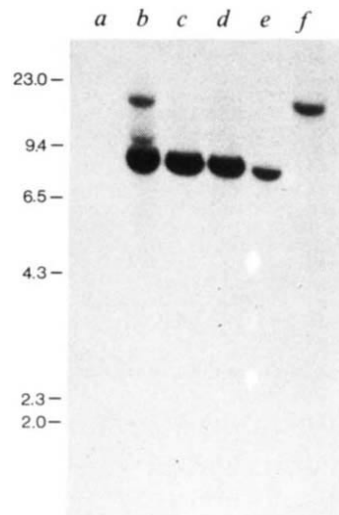


Fig. 2 Presence of the human *N-ras* sequence in primary and secondary transfection foci. Cellular DNA (15 μ g) was digested overnight with *Eco*RI and a Southern blot performed as described in Fig. 1 legend except that the final wash was at 62 °C. The probe was an *Eco*RI-*Sst*I subfragment of the human *N-ras* 8.8-kb *Eco*RI fragment⁹, nick-translated to a specific activity of 4×10^8 c.p.m. per μ g of DNA. Cellular DNA was from: a, NIH 3T3 cells; b, primary focus; c-f, secondary foci as in Fig. 1.

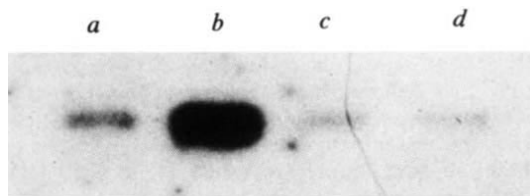


Fig. 3 Lack of *myc* gene amplification in AML DNA. Aliquots of cellular DNA (15 μ g) were digested overnight with *Sst*I and a Southern blot was performed as described in Fig. 1 legend. The probe was pMC41-3RC, a 1.4-kb fragment¹⁴ of the human *myc* gene cloned into pBR322. Specific activity was 5×10^8 c.p.m. per μ g DNA. Cellular DNA was from: a, normal human spleen; b, human promyelocytic leukaemia HL-60; c, bone marrow from AML patient; d, fibroblasts derived from AML patient. The size of the fragment shown is 2.8 kb.

background values (compare Table 1). However, sequencing data from the cloned *N-ras* alleles will be needed to establish more firmly that *N-ras* activation in this patient was indeed a somatic event. It will be interesting to see whether this activation involves similar mutational events to those reported for the *H-ras* gene, namely a single base pair change altering either amino acid 12 (refs 23-25) or 61 (ref. 26) of the corresponding p21 protein. In addition, the present demonstration of an activated *N-ras* gene from an AML patient occurring in the absence of detectable *myc* gene alteration calls for an extensive study on the status of the *N-ras* gene and its expression in acute leukaemias.

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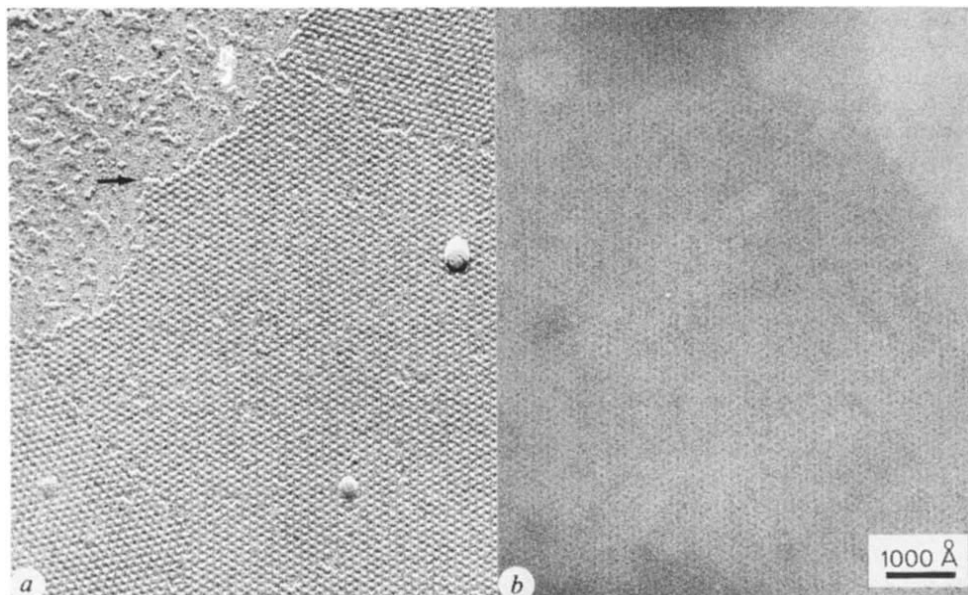
Three-dimensional structure of the light-harvesting chlorophyll *a/b*-protein complex

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Photosynthesis in the chloroplasts of green plants is carried out by a highly organized system of membranes. Chlorophyll-protein complexes in the photosynthetic membrane perform specialized functions which result in the biochemical fixation of solar energy. The light-harvesting chlorophyll *a/b* protein complex (LHC) has a key role in this process^{1,2}. A striking property of the LHC that distinguishes it from other chlorophyll-protein complexes and makes it suitable for structural studies by Fourier methods is its tendency to form crystalline arrays *in vitro*³. I report here the three-dimensional structure of pea LHC determined at 16 Å resolution by electron microscopy of two-dimensional crystals and image analysis. A three-dimensional map shows that the LHC is a highly asymmetric transmembrane protein composed of three structurally equivalent monomers. The large surface area exposed on one side of the trimeric complex suggests a functional role in membrane interaction. The ability of LHC isolated from widely different plant species to form similar crystalline arrays^{4–6} suggests that the structure reported here is of general significance.

Fig. 1 Two-dimensional crystals of the LHC from pea chloroplasts: *a*, freeze-dried and shadowed with platinum at an angle of 45°; *b*, negatively stained with a 2% (w/v) uranyl acetate solution containing 0.1% (w/v) Triton X-100 (Sigma). The shadow cast at the edge of the crystalline sheet in *a* (arrow) indicates a thickness of 60 Å. The surface relief of the crystals shows a hexagonal array of holes measuring roughly 90 Å across which span the crystalline sheets. The holes appear as dark pools of stain in negatively contrasted sheets (*b*). The LHC was isolated from pea chloroplasts as described elsewhere⁴. KCl was added to the fluorescent density gradient fraction to a final concentration of 200 mM. The microcrystalline precipitate was pelleted after 3–4 min at room temperature and redissolved in 0.2% (w/v) unbuffered Triton X-100. Two-dimensional crystals were obtained by dialysis of the purified gradient fraction against 300 mM KCl, 10 mM Tris-HCl pH 6.9, 0.1% (w/v) Triton X-100 and 1 mM Na₂S₂O₃ for ~1 h at 32–39 °C.



Apart from its function as a molecular antenna that harvests solar energy, the LHC regulates the transfer of excitation energy to the reaction centres of photosystems I and II, depending on the phosphorylation of the LHC apoprotein². The complex also mediates the interaction of thylakoid membranes which leads to the formation of stacks of apposed membrane vesicles, the chloroplast grana. This process is triggered by cations and resembles the formation of crystalline arrays *in vitro* which occurs spontaneously on addition of cations to the detergent-solubilized complex⁷. By controlled dialysis against moderate salt concentrations it is possible to obtain extensive, highly ordered two-dimensional crystals of the LHC. These crystalline sheets have a hexagonal lattice with a 125 Å unit cell dimension⁸ and belong to the P321 space group. They are composed of interdigitating triangular units alternating in orientation to the plane of the sheet⁴.

Specimens of crystalline sheets shadowed with heavy metal to reveal their surface structure show a hexagonal array of holes ~90 Å in diameter (Fig. 1*a*). The holes span the two-dimensional crystal, indicating the absence of a continuous lipid bilayer. The shadow cast in the holes and at the edges shows that the sheets are ~60 Å thick and therefore are single layers of molecules, an important prerequisite for this type of structural analysis. When contrasted with negative stain, the crystals resemble a honeycomb with the stain-excluding protein surrounding dark pools of contrasting agent within the holes (Fig. 1*b*).

Two-dimensional crystals contrasted with the negative stain, uranyl acetate, were used to determine the three-dimensional structure of the LHC in a way similar to that described by Henderson and Unwin⁹. This method takes advantage of the fact that the Fourier transform of an electron micrograph of a two-dimensional crystal is a central section through the three-dimensional Fourier transform of the crystal, that is, through a lattice of lines in reciprocal space which are perpendicular to the plane of the crystal. By combining the Fourier transforms of a sufficient number of different, tilted views it is thus possible to measure the amplitudes and phases of Fourier terms along each lattice line. As in X-ray crystallography, an inverse Fourier transform then yields a three-dimensional map of the structure. In this analysis, amplitude and phase data were obtained by computing the Fourier transforms of well-ordered, densitometered areas of 43 electron micrographs of LHC crystals tilted up to 82° in the electron microscope. Fourier terms at the points of the reciprocal lattice (where the transform plane intersects the lattice lines) were collected and combined, using

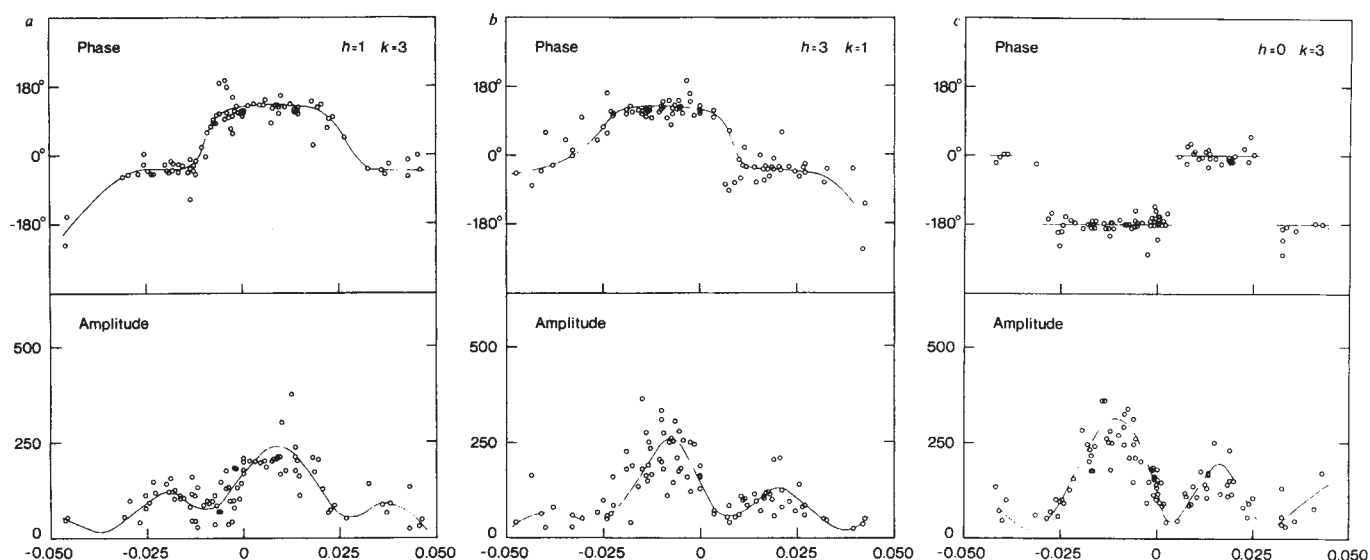
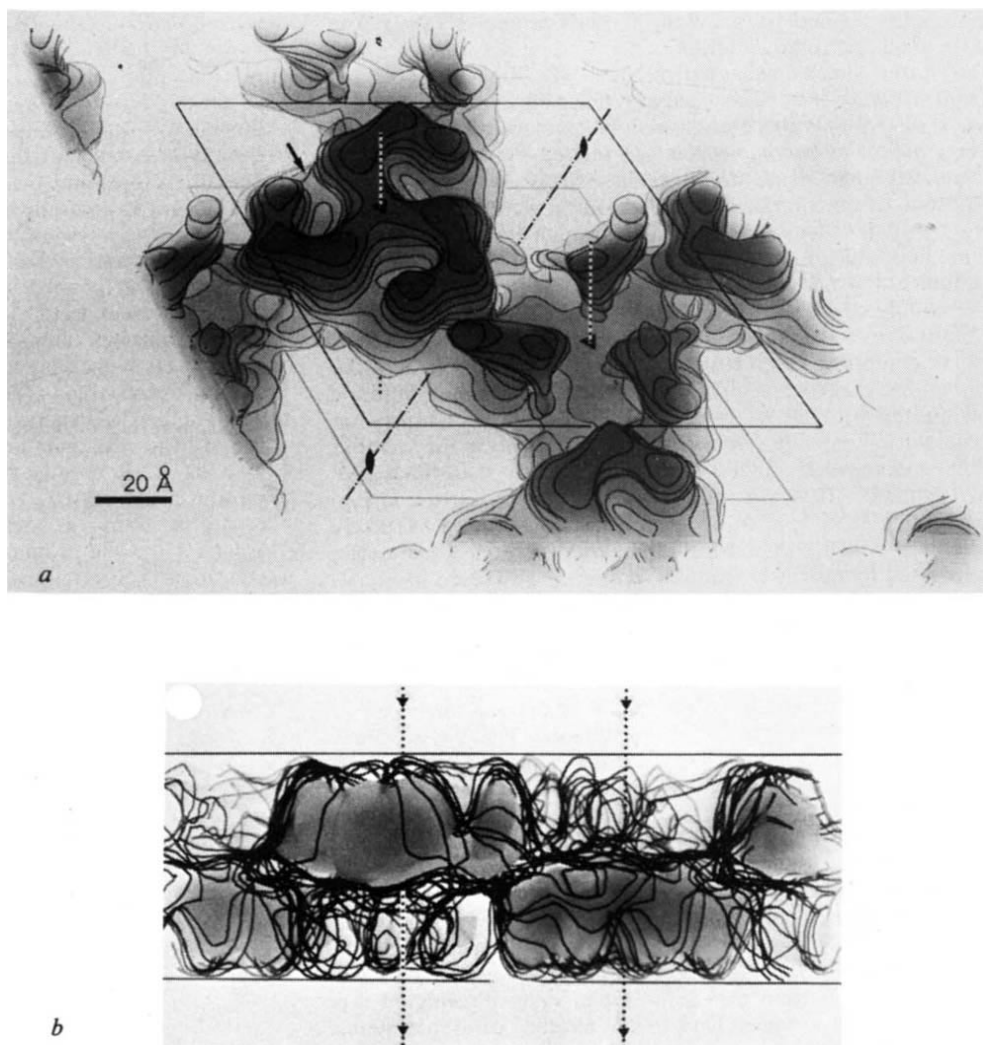


Fig. 2 Amplitude and phase of Fourier terms with Miller indices: *a*, $h=1$, $k=3$; *b*, $h=3$, $k=1$; and *c*, $h=0$, $k=3$ as a function of the reciprocal space coordinate, z^* . The apparent mutual mirror symmetry of pairs of lattice lines (h, k) and (k, h) (for example, *a* and *b*) and the real phase angles of 0° and 180° in the entire z^* range for axial lattice lines (for example *c*) indicate P321 symmetry. Data to a resolution of $16 \text{ \AA}$ were collected at reciprocal lattice points on computed Fourier transforms of 36 images in six tilt series of five negatively stained two-dimensional LHC crystals, with tilt angles ranging from 0° to 62° . Seven images of crystalline sheets recorded at tilt angles between 62° and 82° were incorporated in the final data set. Data were merged using the 3-fold symmetry of the two-dimensional crystals in projection. The phase residual averaged over all images with tilt angles $< 62^\circ$ was 19.3° when no symmetry was imposed (P1 space group), 19.2° for P3, and 19.4° for P321 symmetry, confirming that the highest symmetry, P321, applied.

Fig. 3 Three-dimensional map of the LHC viewed from above (*a*) and edge-on (*b*) drawn to the same scale. The map shows two complexes in the unit cell. A 3-fold symmetry axis ($\cdots$) runs centrally through each complex. The two trimeric LHC molecules are related by a 2-fold symmetry axis ($\cdots$) so that adjacent complexes alternate in orientation perpendicular to the plane of the crystalline sheet. Different structural detail is exposed on the two external surfaces of the complex, which is a highly asymmetric, integral trans-membrane protein. The edge-on view (*b*) in a direction perpendicular to the unit cell edge at the front of *a* indicates the vertical distribution of density within the complex. The main mass is found near the side of the complex which in the top view is characterized by three hook-like arms extending around three darkly staining gaps (arrow). The thickness of the LHC trimer and of the crystalline sheets is $\sim 59 \text{ \AA}$. The map was generated from structure factors sampled at regular intervals of $0.005 \text{ \AA}^{-1}$ without imposing 2-fold symmetry. The distance between sections is $3.125 \text{ \AA}$ in the top view and $3.38 \text{ \AA}$ in the edge-on view. Contour lines were drawn at the first positive level of a linear 0 to 9 scale. Areas within the first (*a*) and third (*b*) contour level respectively are shown as opaque.



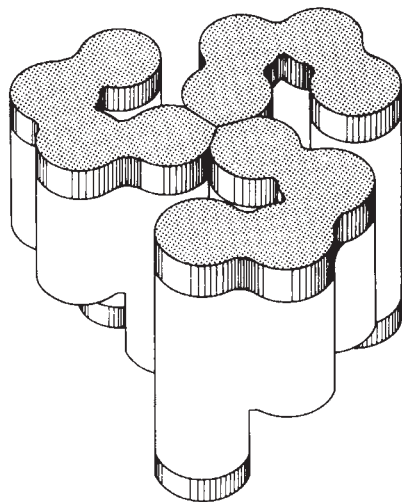


Fig. 4 Schematic drawing of one light-harvesting complex as it appears from the three-dimensional map (Fig. 3). The 3-fold symmetry of the complex indicates that it is a trimer, composed of three structurally equivalent subunits. The shaded regions show the part of the complex that would protrude from a hypothetical 45-Å lipid bilayer symmetrically positioned in the two-dimensional crystal. The platform at the top of the complex suggests a functional role in membrane interaction through van der Waals' forces between LHC molecules in stacked membranes.

the 3-fold symmetry of the crystals, to map out the variation of amplitudes and phases along the lattice lines (Fig. 2). The data extended to a resolution of 16 Å.

A three-dimensional map of the LHC (Fig. 3), generated from structure factors sampled at regular intervals along lattice lines, shows that there are two LHC molecules per unit cell (Fig. 3a). The two molecules are related by a 2-fold axis, a symmetry element of the crystalline sheets that form *in vitro*. In the native membrane, all LHC molecules face the same way up¹⁰. A 3-fold axis runs vertically through the centre of each complex, indicating that LHC is a trimer, composed of three subunits in symmetry-related positions. The trimeric nature of the complex has also been suggested by gel electrophoresis¹¹. The two trimers in the unit cell represent the two main aspects of the complex as seen from above and below the plane of the crystalline sheet, respectively. Clearly, LHC is an asymmetric membrane protein, exposing different structural features on both sides. One side (termed the top side) shows three hook-shaped, somewhat angular lobes separated by stain-filled gaps that extend ~20 Å vertically into the complex (arrow in Fig. 3a). Each lobe appears to be composed of three vertically oriented, roughly cylindrical domains of 15–20 Å diameter, connected by narrower bridging regions as indicated in Fig. 4. The other side shows three oblong protrusions which are continuous with the hook-shaped lobes.

An edge-on view of the three-dimensional map (Fig. 3b) shows that the thickness of the crystalline sheet and of the LHC trimer is ~59 Å, which agrees well with measurements made on stacked sheets¹² and by heavy metal shadowing. Seen edge-on, the complex appears highly asymmetric. Roughly half way up the trimer there appears to be a change in density. The less dense region at the bottom of the complex is taken up by the three elongated protrusions. The dense region which accounts for 80% of the stain-excluding volume consists of the three hook-shaped lobes. The lobes appear to form a rigid platform at the top of the complex which presumably is exposed at the surface of the thylakoid membrane. Immunological studies¹⁰ have shown that both sides of the complex protrude from the native membrane and expose different antigenic sites on the two membrane surfaces. Due to the absence of a continuous lipid or detergent bilayer in the two-dimensional crystals used here, the three-dimensional map does not show by how much

the complex protrudes on the two sides, but, given a bilayer thickness of 45–50 Å (ref. 12), the LHC cannot protrude by more than 10–15 Å (see Fig. 4).

An effect of the pronounced asymmetry of the LHC is a large difference in surface area exposed on both sides. The area exposed on the top side is three or four times larger than that exposed on the opposite side, and roughly twice as large as that exposed by a cylindrical molecule of the same height and volume. It is suggested that the extensive platform at the top of the LHC molecules has a functional role in membrane interaction. The repeat distance of stacked sheets, 56–58 Å (ref. 4), shows that complexes in adjacent membranes can approach one another close enough for van der Waals' forces to take effect¹³. The flat top surface of the LHC molecules seems highly suited for this purpose. Another possible functional role of this part of the molecule is that of a scaffold for orienting and arranging the antenna chlorophylls to cover a large surface area. The three protrusions on the other side could serve to anchor the molecule in the membrane and to make contact with the chlorophyll-protein complexes of photosystem II which are closely associated with the LHC¹⁴.

The purified pea LHC used in this study contains roughly equal amounts of two different, largely homologous polypeptides of 25,000 and 27,000 apparent molecular weight^{4,15} which each bind chlorophyll *a* and *b*. The total number of chlorophyll molecules attached to one polypeptide is in dispute, with reported values ranging from 6 (ref. 16) to 11 (ref. 17). The polypeptide patterns of LHCs isolated from other sources are often more diverse, containing up to four different components in the same molecular weight range. The larger polypeptide of pea LHC has recently been sequenced, giving an exact molecular weight of 28,512 (ref. 18). An estimate of the stain-excluding volume of the trimer at the selected contour level gave a molecular weight of ~100,000, using a specific volume of 1.3 Å³ per dalton derived from the density of bacteriorhodopsin⁹. Allowing for bound chlorophyll, this value suggests that the trimer is composed of three rather than six polypeptides. It seems, therefore, that the two different polypeptides found in pea LHC are structurally equivalent and interchangeable in the trimer, in a way similar to isoenzymes such as lactate dehydrogenase in which two different polypeptides combine to form tetramers of all possible permutations¹⁹. Evidence indicating that the different LHC polypeptides are products of a small multigene family¹⁸ supports this view. LHCs isolated from widely different species such as spinach⁵ and barley⁶ have very similar properties and form crystalline arrays similar to those studied here. The structure reported here may therefore prove to be valid for the main antenna protein of all green plants.

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Thermodynamics of the B-Z transition in superhelical DNA

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One of the most exciting events in recent years in molecular biology was the discovery of the left-handed Z form of the DNA double helix. Originally found in linear self-complementary d(GC)_x·d(GC)_x polymers¹ and oligomers² in non-physiological conditions (a rather high salt concentration), it was recently shown to be easily enough adopted in physiological conditions when purine-pyrimidine sequences are inserted into superhelical DNA³⁻⁹. From such a system, superhelical DNA carrying an artificial purine-pyrimidine insert, we can obtain data allowing the determination of the energy of the junction between the B and Z stretches, F_j , and the free energy change ΔF_{BZ} per base pair (bp). We present here a simple thermodynamic consideration of the B-Z transition in such a system. By applying the results to experimental data³⁻⁹ we have shown that the thermodynamic parameters for both sequences studied so far (d(GC)_x·d(GC)_x and d(GT)_x·d(AC)_x) are similar and equal to $F_j = 4-5$ kcal per mol per junction and $\Delta F_{BZ} = 0.5 \pm 0.7$ kcal per mol per bp.

We consider closed circular DNA, N bp long, carrying an insert of n bp which is potentially capable of adopting the Z conformation, that is, sequences such as d(GC)_x·d(GC)_x, d(GT)_x·d(AC)_x, and so on.

Note that by n we denote the total number of bp in the insert, that is, $n = 2x$.

Generally speaking, we have to allow for the B-Z transition in our statistical-mechanical treatment of superhelical DNA along with open regions and cruciform structures¹⁰⁻¹³ because all these alterations of the B form compete for the superhelix energy. We do that elsewhere¹⁴. Here we present a simple thermodynamic treatment based on the assumption that DNA may experience only the B-Z transition in its artificial purine-pyrimidine insert.

As the negative superhelicity increases, the insert adopts the Z form. In general, this process consists of two stages. At the first stage some fraction of the insert, m_0 bp long, flips from the B to Z form at some superhelix density value σ_0 . Then, if $m_0 < n$, the rest of the base pairs in the insert would adopt the Z conformation gradually as the negative superhelix density increases further. The flipping occurs when the free energy increase due to the formation of the m -bp Z-form fragment is balanced by the energy decrease due to the relaxation of the strain in the superhelical DNA:

$$2F_j^* + m\Delta F_{BZ} + \Delta G_m = 0 \quad (1)$$

Here F_j^* is the effective energy of the junction between B and Z forms, ΔF_{BZ} is the free energy change per bp for the B-Z transition. ΔG_m is the change in superhelix energy when m bp are converted from the B to Z form, which is known to be^{5,15,16}:

$$\Delta G_m = 10RTN \left[\left(\sigma + \frac{1.8m}{N} \right)^2 - \sigma^2 \right] \quad (2)$$

Here σ is the titratable superhelix density value in DNA, and the factor 1.8 appears because the number of bp per helical turn is assumed to be 10.5 in the B form and 12 in the Z form^{2,5}. Substituting equation (2) into equation (1) we obtain:

$$32.4RTm^2 + mN(\Delta F_{BZ} + 36RT\sigma) + 2NF_j^* = 0 \quad (3)$$

The minimal value of $-\sigma$ for which equation (3) has a real root with respect to m defines the superhelix density of flipping,

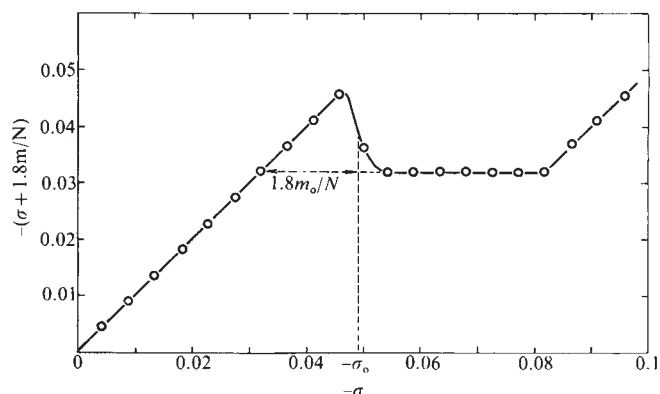


Fig. 1 The data of Haniford and Pulleyblank⁸ presented in a form suitable for theoretical analysis. The value $\sigma + 1.8m/N$ is determined directly from the scaled experimental value of DNA mobility in gel. The value of σ_0 corresponds to the middle point in the flipping of the m_0 -bp stretch.

σ_0 . This value is determined by setting the discriminant of quadratic equation (3) at zero, leading to:

$$\sigma_0 = -\frac{1}{36RT} \left(\Delta F_{BZ} + 11.4 \sqrt{\frac{2F_j^*RT}{N}} \right) \quad (4)$$

The number of bp that flip at this superhelix density:

$$m_0 = 0.175 \sqrt{\frac{2F_j^*N}{RT}} \quad (5)$$

Equations (4) and (5) are valid only if $m_0 < n$. If they lead to an m_0 value larger than n it means that the whole insert flips to the Z form at some value, σ_n ($|\sigma_n| > |\sigma_0|$) and this value is determined by equation (3) with m replaced by n . In the case of $m_0 < n$ the flipping is followed by a gradual growth of the Z form stretch with increasing negative superhelicity. For m values within the interval $m_0 < m \leq n$ the following equation holds:

$$\Delta F_{BZ} + 36RT \left(\sigma + 1.8 \frac{m}{N} \right) = 0 \quad (6)$$

Equation (6) reflects the fact that the energy increase due to the transition of a base pair from the B to Z form should be balanced by the energy decrease due to the decrease of the superhelix strain. Equation (6) shows that

$$\sigma + 1.8 \frac{m}{N} = \text{const. for } m_0 < m \leq n \quad (7)$$

Before comparing the results with experimental data we must emphasize that our treatment was oversimplified in one important point. We have neglected the entropic terms that originate from the fact that a number of states with the same or almost the same energy correspond to a given value of m (when $m < n$). Most probably all positions of the originally flipping stretch of m_0 bp are energetically equivalent while it is within the n -bp insert. We have performed rigorous calculations based on the partition function. The complete results will be presented at length elsewhere¹⁴. The general situation has proved to be rather complicated. It is simple if m_0 is small compared with n , in which case the above equations remain valid with

$$F_j^* = F_j + \Delta \quad (8)$$

The value of Δ is a weak function of the parameters and may be considered as a constant $\Delta = -1.8$ kcal per mol. When $m_0 \geq n$, the entropic terms may be practically neglected, that is, $F_j^* = F_j$.

Turning to the experimental situation, we see that both behaviours predicted by the above theoretical analysis were really observed. In most cases the insert flipped as a whole³⁻⁷ but Haniford and Pulleyblank⁸ have recently observed the flipping of a part of the insert followed by the elongation of the Z form stretch with increasing negative superhelix density.

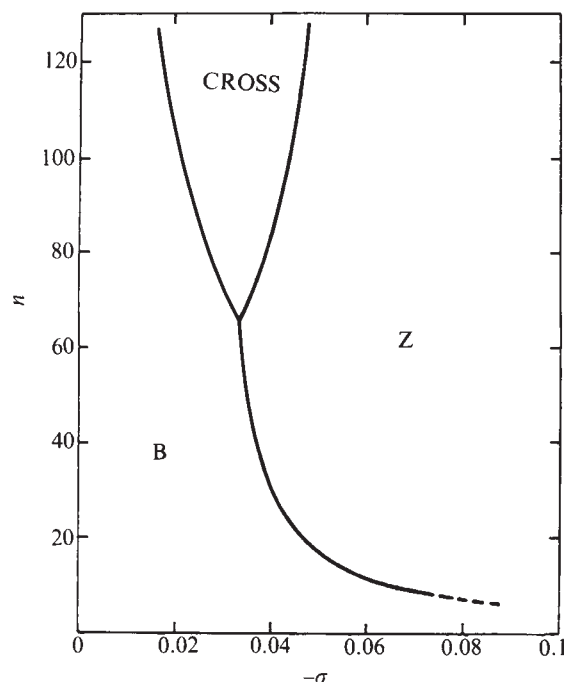


Fig. 2 Phase diagram for an n -bp $d(GC)_x \cdot d(GC)_x$ sequence inserted into long superhelical DNA with superhelix density σ . The calculations have been performed for $F_j = 4.0$, $\Delta F_{BZ}^{GC} = 0.6$, $F_s = 6$ and $\Delta F^{GC} = 2.1$ kcal per mol corresponding to 0.2 M Na^+ .

In their experiments the $d(GT)_{30} \cdot d(AC)_{30}$ sequence was inserted into a plasmid with $N = 2.2 \times 10^3$ and at $\sigma_0 = -0.050$ $m_0 = 22$ bp flipped into the Z form⁸. Then the writhing number remained unchanged until the whole insert adopted the Z conformation. During this stage $\sigma + 1.8m/N = -0.032$ (Fig. 1). Using equations (5) and (6) and the above data, $F_j^* = 2.2$ kcal per mol for junction and $\Delta F_{BZ} = 0.69$ kcal per mol per bp. Substituting these values into eq. (4) we see that they give the observed value of σ_0 . It follows from equation (8) that $F_j = 4.0$ kcal per mol per junction. Since the $-\sigma_0$ value is rather low, the competition with cruciform structures can be neglected. This follows from the control two-dimensional gel electrophoresis pattern in⁸ which shows no discontinuity up to the $-\sigma_0$ value. A cruciform structure would certainly have produced a clear-cut discontinuity in the two-dimensional pattern¹⁷.

Equation (5) shows that an insert would flip as a whole if

$$n \leq 0.175 \left(\frac{2F_j N}{RT} \right)^{1/2} \quad (9)$$

For the most popular plasmid pBR322 ($N = 4.4 \times 10^3$) we conclude, substituting the above value of $F_j = 4.0$ kcal, that the insert would flip as a whole up to $n \approx 40$ bp. This estimate makes it quite clear why most people observed the flipping of whole inserts³⁻⁷. To interpret their data one has to use equation (3) in which m is replaced by n . This equation is similar to the one used by Nordheim *et al.*⁵

It follows from equation (3) that measuring the superhelix density values σ_1 and σ_2 of the B-Z transition for two inserts with lengths n_1 and n_2 one may determine the parameters:

$$F_j = 18RT \left[\sigma_2 - \sigma_1 + \frac{0.9}{N} (n_2 - n_1) \right] \left(\frac{1}{n_1} - \frac{1}{n_2} \right)^{-1} \quad (10)$$

$$\Delta F_{BZ} = 36RT \left[\frac{n_1 \sigma_1 - n_2 \sigma_2}{n_2 - n_1} - \frac{0.9(n_1 + n_2)}{N} \right] \quad (11)$$

Substituting the experimental values⁵ ($n_1 = 14$, $n_2 = 32$, $\sigma_1 = -0.072$, $\sigma_2 = -0.048$) into equation (10) we obtain $F_j = 7.5$ kcal per mol in agreement with the original paper⁵ but contradicting

the above estimation. This may be explained by cruciform formation in the carrier pBR322 DNA between superhelix densities σ_1 and σ_2 . Indeed, Singleton¹⁸ has recently shown that in pBR322 DNA a 35 bp palindrome with the centre in position 3,065 assumes a cruciform. If in the conditions described by Nordheim *et al.*⁵ it is formed within the superhelix density interval $\sigma_1 < \sigma < \sigma_2$ then we should replace in equations (10) and (11) the σ_1 value by the value $\sigma_1^* = \sigma_1 + n_+/N$ (n_+ is the number of bp in the palindrome¹³), then $\sigma_1^* = -0.064$ and equations (10) and (11) yield the values $F_j = 5.3$ kcal per mol and $\Delta F_{BZ} = 0.56$ kcal per mol. This example shows that the behaviour of comparatively short inserts that flip at high negative superhelix densities may be seriously affected by the cruciform formation in the carrier DNA¹⁴.

The remaining difference between the values of parameters obtained for $d(GC)_x \cdot d(GC)_x$ and $d(GT)_x \cdot d(AC)_x$ inserts can hardly be regarded as significant and most probably reflects the accuracy of our present-day knowledge of the parameters. Other estimations of the same parameters have very recently led to quite similar results^{19,20}.

Having the parameters of the B-Z transition, we can predict the behaviour of a purine-pyrimidine insert for different n . If the sequence of the insert is not self-complementary, the behaviour is governed by the above equations. However, a self-complementary sequence (such as $d(GC)_x \cdot d(GC)_x$) can adopt a cruciform structure as well as a Z form. So in this case we have to consider the equilibrium between three possible states: the B form, the Z form, and the cruciform. For the sake of simplicity we will assume that N is very large and as a result m_0 is larger than n for all values of n under consideration. The free energies of the insert's transition from the B to Z form and from the B form to the cruciform are

$$F_z = n(\Delta F_{BZ} + 36RT\sigma) + 2F_j \quad (12)$$

$$F_+ = 20RTn\sigma + 3F_s + 4\Delta F^{GC} \quad (13)$$

Equation (12) immediately follows from equation (3) at $N \rightarrow \infty$. In equation (13) the last two terms are the energy of the cruciform formation, that is, the energy of six helix ends, and the energy of the formation of two single-stranded loops comprising four nucleotides each^{10,11,13}. Using equations (12) and (13) we obtain the phase diagram plotted in Fig. 2. The diagram shows that small n values are not favourable for a cruciform structure. However, for n larger than 70 there is an interval of superhelix densities where a cruciform structure proves to be the most stable conformation. This interval grows as n increases.

Note that this behaviour may be difficult to observe because of the very large relaxation time, especially for the cruciforms^{17,21}.

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Anatomy of higher education

Eric Ashby

The Higher Education System: Academic Organization in Cross-National Perspective.

By Burton R. Clark.

University of California Press: 1983. Pp.315. £21.25, \$32.50.

TO SET this book in perspective, here are three incidents from my own experience.

First, dinner at high table in the University College of the Gold Coast (now Ghana) in 1956. The students, wearing gowns, stand as we file in: the master, the senior tutor, the steward, the chaplain. A Latin grace is read. After dinner we retire to the Combination Room and are offered port. The terms, in a nation with a large Muslim and pagan population, were labelled Michaelmas, Lent and Trinity. The purchasing officer was called a manciple.

Second, a faculty discussion in Lovanium University in what was, in the 1950s, the Belgian Congo. Or, to be accurate, a briefing, not a discussion, for the rector was a Napoleonic figure unconcerned with the niceties of democracy. He was appointed from Louvain and was in direct teletype contact with Belgium from his office in Leopoldville. The number of Congolese graduates at that time was pathetically small for a country about to become independent. What curriculum (I asked) had they devised in order to prepare the Congolese to administer their affairs. Exactly as in Louvain, was the reply: a basic course in philosophy, French language and French literature before qualifying for any professional training; *Chanson de Roland* in the first year.

Third, a visit to the University of Nigeria in Nsukka, created in the early 1960s under the sponsorship of Michigan State University. As you come from Enugu you see the university spread below you, on a flat plain below a steep hill, with the central, predominant feature of a sports

stadium. The cafeteria, I found, was crowded with somewhat subdued and perplexed farmers from the surrounding villages, attending a week-end course in poultry nutrition.

These were just the superficial signs of the genetic imprint of Cambridge on Ghana, Louvain on the Congo and Michigan on Nigeria. It permeated curriculum, style of administration, academic values. At first there was scant evidence of adaptation to the local society. This deep imprint of older universities on newer ones is not just a recent phenomenon. In 1551 the Spanish emperor Charles V founded universities in Mexico and Peru with all "the privileges, exemptions, and limitations of the University of Salamanca". Even the order of precedence in the academic procession and the exact procedure at convocations had to be prescribed from Salamanca. In Scottish universities there is a vestige of inheritance from Bologna, when the students elect the Rector. In Cambridge, candidates for the bachelor's degree are presented to the vice-chancellor with the assurance (fortunately delivered in Latin) that they have satisfied their teachers in morality as well as in learning. For centuries the university, like church and parliament, has shown extraordinary resilience and capacity for adaptation — surely a challenge to scholars to analyse how this has happened. Yet, despite shelf-loads of rhetoric about "the Idea of a University" and some good historical accounts of universities in separate countries, very few scholars have risen to it. Burton Clark is one of them. This alone makes his book an important one.

The easy way to approach the task is to

describe, seriatim, the systems of higher education in different countries and then in a final chapter to apply the formula familiar to examinees — "compare and contrast". The result is rarely illuminating and invariably dull. Clark meets the challenge in a more ambitious way. What, he asks, are universities *for*? What do they believe about themselves and what does the so called "layman" (that word is historically significant too) think about them? Where does authority lie in systems of higher education? They have changed over the centuries and by implantation into alien cultures: what have been the incentives, and what have been the impediments, to change?

The raw material for universities is knowledge. They are, Clark declares, institutions for the management of knowledge; research and teaching are the technologies employed in management. But knowledge is not homogeneous. An institution for the management of knowledge brings on to one campus mediaevalists, biochemists, engineers. Why? Mediaevalists can get on perfectly well without biochemists. Are they together simply to share the central heating system and the catering facilities? In the Soviet system much professional education is in staff colleges attached to the appropriate ministries: lawyers in a college run by the ministry of justice, doctors in a college run by the ministry of health. Yet an institution embracing many diverse disciplines remains the standard pattern for higher education.

Clark's painstaking analysis of this question holds no surprises for anyone who has spent a lifetime in academe, but it includes a great deal that is not understood by the politicians and bureaucrats who now have such influence over the future of higher education. He explains how it is much more than basic facilities that bring scholars together into universities. They share certain beliefs; they have common problems of divided loyalty; they cannot work well unless they are allowed to devise for themselves a very peculiar structure of

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

"Institutions for the management of knowledge". Left to right: Clare College and King's College Chapel, University of Cambridge; Stanford University; and the Université Catholique de Louvain.

power and authority, one in which the process of decision-making does not descend in decrees from above but arises on the "shop floor" and percolates upwards, to reach (paradoxically) the desk of the individual who, *de jure*, is the boss of the institution. Prominent among the beliefs is a common purpose: to transmit, by teaching, the orthodoxy of the discipline and to generate, by research, a rigorously controlled system of dissent from orthodoxy. There is a mystique about this and there is intense loyalty to an institution that nurtures this mystique. But these communities of scholars have to come to terms with two often conflicting loyalties. On one hand their loyalty is to the discipline: what matters most to a physicist may be his status among other physicists, and this tempts him to put what he calls "my own work" before the interests of the university in which he serves. On the other hand their loyalty is to the institution and its corporate interests: what matters most may be the educational mission of the community, to produce (as the Oxford don, Mark Pattison, put it) "not a book but a man". "The culture of a discipline even includes idols", writes Clark. In the sociologist's office you see a picture of Max Weber on the wall. Yes, and in the office of a chemist who is also a tutor or a dean you see a picture of Magdalen quadrangle on the wall. It is symbolic of the split-personality that inhabits the academic mind. There are subtle differences among different countries in the emphasis given to these loyalties. An American would talk of belonging to the Yale Class of '24; a German would not state his university, but would say "I was a pupil of Heisenberg"; a graduate from Oxbridge does not even mention his university: he is called "a Clare man" or "a Wadham man".

An institution for the management of knowledge has to solve a very difficult problem in the management of its own members. The scholars are differentiated into dozens of disciplines and sub-disciplines. Those in authority over them cannot possibly tell them what to do; yet someone has to allocate space, cut the financial cake into slices, provide the common services. And someone has to defend the university's interests against pressures from what is (shortsightedly) called "the outside world". Clark describes in detail the patterns of academic authority: the dictatorial professor who has the right of patronage (as is to be found in Italy); the collective control by committees of academics (as is to be found in Britain); the administrators who manage the university

as a sort of holding company for semi-autonomous oligarchies of faculties or colleges (as in California); the limited delegated control under a system managed by the state, where the professors are civil servants (as in France). He sees good and bad in all these patterns and wisely refrains from committing himself to preferences among them.

Finally, Clark discusses change in institutions of higher education. Here, it seems, is another paradox: that they are notoriously the most exasperatingly conservative bodies and yet it is from them that the most revolutionary initiatives for changes in society come. Clark finds this puzzling, but I think the explanation may be quite simple. It is that the initiatives come from the "shop floor", from the research and writing of young people. The resistance comes from the people who have become enmeshed in the administrative network of the place or whose interests

would be endangered by change. The obduracy of the University of London to reform itself is a local British example of this.

Clark's book is written with authority but I must add a warning that his conclusions are at a level of generalization which I found at times hard to follow. I am not a sociologist, so it is not for me to carp about the use of abstract latinized words which to the uninitiated may seem to be jargon. In writing this "layman's" review of it, I may have oversimplified some of Clark's subtle reasoning, but I hope I have made it clear that this is a book which, even if it isn't to be read for pleasure, is certainly to be studied for profit. □

Lord Ashby is a Fellow of Clare College, Cambridge, and has been a professor in the Universities of Sydney and Manchester, and vice-chancellor in the Universities of Queen's, Belfast, and Cambridge.

Archaeology, A to Z

Colin Platt

The Macmillan Dictionary of Archaeology.

Edited by Ruth D. Whitehouse.

Macmillan, London: 1983. Pp.597. £25.

THE emergence of archaeology as an amalgam of several disciplines has been comparatively recent, dating back scarcely further than the 1960s. And it is this crossing of boundaries, still bewildering to many, that is the chief justification for Dr Whitehouse's new *Dictionary*. Handsomely presented in a generous layout, it has the enticing feel of a good work of reference: essential, of course, for instant instruction, but as well suited for a pleasurable browse. How many of us would have been aware, without such aid, that the earliest phase of the North Vietnamese Bronze Age is known to its students as "Go Bong"? On the very same page, we are cross-referred away from Gogo Falls, and are given a good, short account of the goat. Here is pleasure, stimulus and intelligence, all in one.

Nobody need have misgivings about the intended scope of this dictionary. Its field is the wide world; its timespan is four million years; its concern is with techniques as well as evidence. Nevertheless, what precisely were the principles, we are still bound to ask, that guided Ruth Whitehouse and her small team of subject editors in their selection of material for discussion in these pages? In the little she tells us on this fundamental matter, Dr Whitehouse is not reassuring. "I cannot claim", she confesses, "that coverage is even. . . . My own prejudices, and to a lesser extent those of my subject editors, have necessarily prevailed." One of these prejudices — perhaps a considered principle, though we

are not so informed — is that the best archaeologist is necessarily a dead one. Thus David Clarke (d.1976), as one of the pioneers of the New Archaeology which itself gives purpose to this book, gets his entry. But Lewis Binford, very much more influential in the same field as Clarke precisely because he remains alive and active, does not. The editors have been spared embarrassing decisions, but only to the impoverishment of their text.

In the same way, other omissions would have been easier to accept if more trouble had been taken to explain them. Of course, this is a familiar game among critics of dictionaries, but so much the more reason to anticipate it. Why Repton and not Deerhurst? Why Krak des Chevaliers and not Sahyun? Why San Vincenzo al Volturno and not Monte Cassino? In each case, the answer seems to be that there have been excavations (or research programmes) on these sites known personally to one of the subject editors of this volume. And increasingly, as we come to follow through our individual specialisms, the *Macmillan Dictionary of Archaeology* begins to look narrowly based. Why, to go back to the general editor's opening apologia, do the compilers' prejudices, in a work of reference of this kind, "necessarily" have to prevail? They are a clannish group in which marital pairings and former scholarly cooperations are prominent. Could it be that there were just not enough of them from the start?

Fortunately, the preoccupations of Ruth Whitehouse's "team of international experts" are very likely to be those of the great mass of users of this dictionary. Nor is there any doubt that there is much of real value in these pages, clearly and professionally expressed. Mutton dressed as lamb, perhaps. But still good food. □

Colin Platt is a Professor of History at the University of Southampton.

Surface Physics

Dr J.E. Inglesfield asks us to point out that the title to his review of M. Prutton's *Surface Physics*, 2nd Edn (*Nature* 307, 190; 1984) was chosen not by him but in the *Nature* office. No slight upon the book was intended by the title "Superficial science".

Editor, *Nature*.

Newton of promise

C.W. Kilmister

Certain Philosophical Questions: Newton's Trinity Notebook.

By J.E. McGuire and Martin Tamny.

Cambridge University Press: 1983.

Pp.519. £50, \$84.50.

WE now know a great deal more about Newton than we did 30 years ago; this is mainly the result of Whiteside's magnificent edition of the papers, with the Halls' collection of the letters coming a close second*. As a result we can see the mistakes as well as the triumphs of a genius, and insights that, for one reason or another, never saw the light of day.

But are we any nearer to understanding this most astonishing of men? Perhaps a little, but because there is still so much left to understand one should hesitate long before relegating a work such as this to the "Newton's laundry book" category. And indeed such a judgement would be wholly unjust. In 1661, at the age of 18, Newton began to keep notes about his studies in Cambridge in a notebook which is in the University Library. But the *Questiones quaedam philosophicae* are (English) notes well on in the book and so almost certainly date from late March 1664 to late 1665. Since 1666 was to be, by his own confession, Newton's *annus mirabilis*, these notes on scientific matters could not fail to be of interest.

The book has been produced with all the care that American scholarship can provide, and the finished work is one of considerable beauty. Two-thirds of it is taken up by an essay describing the *Questiones* in the context of Newton's thought and of other thought of the time, and the remaining third presents, on facing pages, the transcription of the notebook and a modern English version (in which even Newton's sketches are carefully re-drawn). So there is something for everyone. The historian and scholar have an important source presented in meticulous detail. The scientist can read the modern English and see Newton grappling with a variety of questions, some a consequence of the theoretical background with which he had been saddled by his university course, some of a much more modern kind.

He begins with a discussion of atomism

and the "first matter", concluding that there must be atoms, finite in magnitude, and that as a consequence the "world" (i.e. the Universe) must be composed of a finite number of them, since "an infinite number of finite parts cannot be finite". This discussion partakes of an Aristotelian flavour in the arguments rejecting the possibilities of mathematical points serving as atoms. And Newton goes on to infer that, to discuss motion, there must be

a least distance, a least progression in motion, and a least degree of time. Lay two globes together so close that they cannot come any nearer without touching — that is the least distance. Let them be moved together — that is

the water is moved from behind it with but a small force . . .".

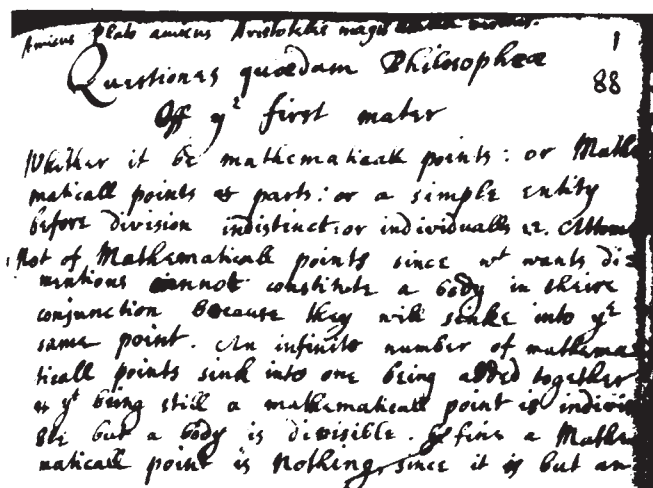
There follows a varied selection of queries — on reflection of light, on atmospheric pressure, "What angle ought a windmill sail make with the wind", on water and salt, on magnetic attraction and electrical attraction, and so to much more on light, vision and colours. Even questions of memory, imagination and sympathy are touched on, succeeded almost at once by consideration of the character of oils, meteors, minerals and the tides. Here, as well as theoretical aspects of the vortex theory, there are notes about the reversing of the tides between Harris and Uist and about the alleged difference in speed of the Danube "as is perceived by the motion and noise of the clackers in mills" between six and midnight though "there is no ebb nor flow, the water keeping at a constant height".

Ever and again violent motion and motion itself return. Newton asks "Whether there can be motion in a vacuum" (since an earlier discussion interpreted the relative character of motion very strictly in terms of adjacent bodies). The argument concludes "If this going of c to d be not motion I ask what it is. But this is only to strive about terms, and if it pleases you not to call it motion call it what you will, but it is that which we aimed to prove". There are many astronomical obser-

vations and discussions, mostly of comets, before a return to motion yet again and then more optics. So it goes on, with notes on God and the soul, before various chemical queries come all together — genuine chemistry rather than alchemy, such as: "Alcalizate salts are wont to precipitate what acid salts dissolve".

So where does the notebook show us Newton standing? We see a man with at least part of one foot still in the Middle Ages, still needing to liberate himself from the weakening clutch of Aristotle; a man who has studied Descartes and is evidently not too happy about vortices. But above all we discern an enquiring mind, compulsively interested in everything around him or reported to him. In some ways a more human figure emerges. The authors tell us that "Newton's copy of Diogenes is extensively dog-eared . . .". As Newton used the technique of dog-earing, a dog-ear was used not only to mark a page but often to mark a place on a page". But the mystery is still there. The author of the notebook is the young man of promise. The Newton of 1666 still seems a different person. □

C.W. Kilmister is Professor of Mathematics at King's College, University of London, and a past President of the British Society for the History of Mathematics.



In his own hand — the title page of the "Questiones", consisting of Newton's notes on Walter Charleton's consideration of the nature of first matter in "Physiologia Epicuro-Gassendo-Charltoniana".

the least degree of motion and it is performed in the least part of time.

So here was someone still far from understanding the use, let alone the nature, of fluxion or fluent, but at the same time preparing the ground for the seed. He returns to such problems later, evidently still worried, but without resolving them. This really summarizes the character of the whole book.

The next discussion is of the Sun, stars and planets: "Whether the Sun moves the vortex about (as Descartes's will) by his beams. . . . How it is that the Sun turns on his axis". Then a quotation "Hebrews, Chapter 1, Verse 2, God made the worlds by his son . . ." is followed shortly after by "On Saturday, December 10, 1664, by a subtle observation I found the distance of a comet from the centre of the Moon to be 9° 48' . . .".

The properties of matter take Newton's attention next, including rarity, density, perspicuity, opacity, gravity and levity. He notes that "the matter causing gravity must pass through all the pores of a body". This is followed by a detailed discussion of the behaviour of this matter as it streams down to the Earth and back again. Then, quite soon, "you may observe in water that a thing moved in it does carry along with it the water behind it, as in a cone, or at least

*The *Mathematical Papers of Isaac Newton* (in 8 volumes). Edited by D.T. Whiteside. Cambridge University Press: 1967–1982. The *Correspondence of Isaac Newton* (in 7 volumes). Edited by A.R. and M.B. Hall. Cambridge University Press: 1959–1977.

Experiments in the gauge revolution

C.H. Llewellyn Smith

Weak Interactions of Leptons and Quarks.

By E.D. Commins and P.H. Bucksbaum.

Cambridge University Press: 1983.
Pp.473. Hbk £37.50, \$69.50;
pbk £16.50, \$27.95.

FOLLOWING the discovery of parity violation in β decay in 1957, it was soon found that a wide range of "weak" decay processes can be described by a simple "phenomenological" theory. The pejorative adjective indicates that this theory is not mathematically consistent and can only work in a limited energy domain. In 1971, 't Hooft showed that consistency could be obtained in gauge theories of the type discussed earlier by Weinberg, Salam, Glashow and others, which have the great attraction of unifying weak and electromagnetic interactions. There was then no experimental evidence for such theories but their revolutionary potential was sufficient to stem the flow of books on weak interactions which had appeared during the 1960s.

Confidence in gauge theories built up as their predictions were gradually confirmed, beginning with the discovery of "neutral current" neutrino interactions at CERN in 1973 and culminating in the discovery of the W and Z bosons — the carriers of the weak force — with the predicted masses at CERN last year. Reflecting these developments, textbooks which incorporate or describe the "gauge revolution" have begun to appear in the past few years. This one, which is suitable for graduate students or as a reference work for researchers, is the first of the new generation of books written from an experimental standpoint in which crucial experiments are described. As well as the traditional topics, there are chapters on most of the subjects which ought now to be in such books — high-energy neutrino interactions, neutrino masses and oscillations, parity non-conservation in eN and NN interactions (including in atoms — the authors' speciality) and neutrinos in astrophysics.

Turning to omissions, it is odd that there is no derivation — or even statement — of the Fierz transformation, although it is frequently used. The omission of a proper discussion of current algebra and chiral symmetry is more serious. The Adler-Weisberger and Goldberger-Treiman relations appear (although the latter is confused with pole dominance of the pseudo-scalar form factor and we are not told whether the real relation works). Discussion of the Callan-Treiman relation and predictions for slope parameters in K

$\rightarrow 3\pi$ surely also deserved some space. On the other hand, the "abbreviated and superficial" discussion of path integrals will help neither the novice nor the expert and could have been left out. The authors nowhere really use or need the full Feynman rules which they derive using this technique (although some discussion of testable higher-order predictions of gauge theories might have been desirable).

A more serious criticism is that the book contains several theoretical remarks which are dubious or misleading. Two of the more subtle examples must suffice. It is not true that Lagrangians "satisfy the Euler Lagrange equations" or that current conservation is in general equivalent to invariance of the Lagrangian (the whole question of auxiliary fields in supersymmetry bears witness to this). The mass-matrix for the K^0 - $\bar{K}^0$ system does not follow from the Schrödinger equation given (although the appearance of the word "effective" perhaps provides an alibi) — a derivation involves the Wigner-Weisskopf

theory of decaying states. A final point is that it seems perverse in a book largely devoted to interactions of neutrinos to normalize spinors in a way which is singular for massless particles.

Despite its faults, this is not a bad book. There are better introductions to gauge theories, such as *Gauge Theories in Particle Physics* by I.J.R. Aitchison and A.J.G. Hey (Adam Hilger, 1982), and clearer discussions of the phenomenology of weak interactions, for example in L.B. Okun's *Leptons and Quarks* (North-Holland, 1982) or T.D. Lee's monumental *Particle Physics and Introduction to Field Theory* (Harwood, 1981). However, this is the only up-to-date book on weak interactions which includes a discussion of the experiments and the data, and I would therefore advise students to read it and librarians to buy it. □

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Exciting stuff

F.W.J. Teale

Principles of Fluorescence Spectroscopy.

By Joseph R. Lakowicz.

Plenum: 1983. Pp.496. \$32.50, £25.

EVER the most cursory glance over the literature of biochemistry, biotechnology and clinical medicine confirms the continuing growth in the exploitation of fluorescence techniques. How then should uninitiated researchers equip themselves to join the enlightened throng? Only the most dedicated can afford the time necessary to gain experience in a recognized "fluorescence" laboratory. More ordinary mortals have recourse to reading reviews, specialized articles and older classic books and papers selected from a large and diffuse literature. This can be a hard, unrewarding slog for the newcomer, and there has long been a need for a suitable primer in this field.

Joseph Lakowicz has attempted to remedy this need by bringing within a single monograph the basic introduction, essential theory and working practice of the most important aspects of fluorescence spectroscopy, at least as encountered in the life sciences. The reader has the vicarious benefit both of what is clearly a thorough knowledge of the literature, and of the author's experience in a well-known laboratory since his treatment strongly reflects the influence of Gregorio Weber to whom the book is dedicated. This is no small recommendation in itself. Phase-modulation techniques, after two decades of undeserved and incidental eclipse by pulse-excitation, are rightly restored to pre-eminence, while the phase-dependent

method developed by the author for resolution of heterogeneous emission occupies a complete short chapter. Longer, well-illustrated sections are devoted to the fundamental fluorescence parameters and to a selection of excited-state processes implied by the "spectroscopy" in the title. As the latter suggests, the main emphasis is on fundamental aspects, while examples are taken almost entirely from protein and membrane systems.

A description of the essential instrumentation, calibration methods, common malfunctions and experimental pitfalls comes immediately after a short general introduction. This introduction, however, is barely adequate in conveying the range of molecular information potentially made accessible by appropriate strategies, and an early chance to whet appetites is rather missed. That said, the level of presentation is nicely poised between the superficial and the recondite. So that the reader has some likelihood of pursuing them, references are restricted to major benchmark publications (except for several "unpublished observations", which would have been better dealt with in the text). A useful feature, stressing the didactic intention of the author, is the inclusion of simple problems at the end of most chapters; if attempted, these oblige the reader to understand fully the main thrust of the preceding material. Correct solutions are also provided for reassurance.

Lakowicz's aim in writing this book was to satisfy the requirements of those interested in understanding the principles underlying the various fluorescence techniques encountered in contemporary research. To my mind, he has succeeded. □

F.W.J. Teale is the Reader in Physical Biochemistry in the Department of Biochemistry, University of Birmingham.

Appointments

Professor R.L. Wain has been appointed the first Royal Society Kan Tong-Po visiting professor to Hong Kong. The visiting professor scheme has been set up to allow scientists from the Commonwealth and the United States to spend four to ten months in the universities of Hong Kong, assisting with research and lecturing whilst pursuing their own research interests.

Awards

Applications are invited from young Australians and permanent residents in Australia for the Fogarty International Centre's research fellowships. The awards are designed to allow research in health-related fields in any biological or medical laboratory in the United States. Up to six awards may be made from Australia, and each fellowship lasts for twelve months, although an extension of a year may be considered. Candidates must have achieved a doctoral degree in the health sciences, preferably within the last six years and not more than 10 years ago. A stipend of \$18,000 to \$22,000 is provided according to relevant postdoctoral experience. Further information and application forms are available from the Australian Academy of Science, PO Box 783, Canberra, ACT 2601, Australia.

The British Medical Research Council is looking for candidates to nominate for the NIH international research fellowships of 1985-86. A competition for nomination for these awards will be held by the MRC in June 1984. The research fellowships are awarded for 12 months at a stipend level that ranges from \$16,000 to \$20,000 per year depending on experience. Application forms are available from The Training Awards Group, Medical Research Council, 20 Park Crescent, London W1N 4AL, UK.

The British Institution of Production Engineers has made the following awards: the International Award to **Dr Alan Puckett** of Hughes Aircraft Company; the Mensforth Gold Medal to **Mr Peter Jefferson** of British Aerospace; the Nuffield Award to **Sir Robert Telford** of Marconi Electronics and the Institution Silver Medal to **Mr John Adler**, former chairman of the Institution's northern region.

Foulkes Foundation fellowships have been awarded to **Miss Susan Griffith** of University College, London; **Dr Mary Ninkovic** of the University College Hospital Medical School, London; **Dr**

Deenan Pillay of the University of Newcastle-upon-Tyne Medical School; **Dr Richard Sheaves** of Oxford University; **Dr Kevin Tyler** of Birmingham University and **Mr David Manuel** of Cambridge University.

Meetings

1-3 March, **Symposium on the Clinical Management of the Elderly Patient**, Florida (The Health and Education Council, Franklin Square Hospital, Essex Community College, Baltimore County Health Department, 7201 Rossville Boulevard, Baltimore, Maryland 21237, USA).

6-9 March, **Oceanology International Exhibition and Conference**, Brighton, UK (Oceanology International Conference and Exhibition, Spearhead Exhibitions Ltd, Rowe House, 55-59 Fife Road, Kingston upon Thames, Surrey KT1 1TA, UK).

18-20 March, **Course on Biotechnology in Oil Production**, University of London (Professor V. Moses, School of Biological Sciences, Queen Mary College, Mile End Road, London E1 4NS, UK).

19-21 March, **Workshop on Freezing and Quality Control**, Rockville, USA (Mr David Grounds, Workshop Coordinator, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA).

21 March, **Conference on the Synthetic Applications of Ynes, Enes and Ones**, London (Dr W.B. Turner, Process Development Department, ICI Pharmaceuticals Division, Hurdsfield Industrial Estate, Macclesfield, Cheshire SK10 2NA, UK).

26 March, **International Meeting on Sensory Interactions**, London (Dr H.J.H. MacFie, MRI, Bristol BS18 7DY, UK).

26-28 March, **Workshop on the Use of Computers in Radiology and Oncology**, Geneva (Professor R.J. Berry, Organizing Secretary, Department of Oncology, The Middlesex Hospital Medical School, London W1N 7PN, UK).

26-30 March, **Residential School on Metallo-Organic Chemical Vapour Deposition: Theory and Practice**, Queen Mary College, London (Ms L.A. Hart, Royal Society of Chemistry, 30 Russell Square, London WC1B 5DT, UK).

26-30 March, **International Symposium on the Application of Computers and Mathematics in the Mineral Industries — APCOM**, London (Conference Officer, The Institution of Mining and Metallurgy, 44 Portland Place, London W1N 4BR, UK).

26 March-15 June, **Course in Aflatoxin Analysis and Mycotoxins**, London (Training and Visitors Unit, Tropical Development and Research Institute, 127 Clerkenwell Road, London EC1R 5DB, UK).

27-30 March, **International Conference on Microbiological Quality Assurance in Industry**, Brighton, UK (Beverly Humphrey, Scientific Symposia Ltd, 33-35 Bowling Green Lane, London EC1R ODA, UK).

2-4 April, **Consensus Development Conference on Osteoporosis**, Bethesda, Maryland (Mr Peter Murphy, Prospect Associates, Suite 401, 2115 East Jefferson Street, Rockville, Maryland 20852, USA).

3-5 April, **Symposium on Solids/Liquids Separation Practice and the Influence of New Techniques**, Leeds, UK (Dr Paul Preece, Department of Chemical Engineering, University of Leeds, Leeds LS2 9JT, UK).

3-6 April, **International Conference on Energy Options**, London (The Institution of Electrical Engineers, Savoy Place, London WC2R 0BL, UK).

9-11 April, **International Conference on Simulation for Nuclear Reactor Technology**, Cambridge, UK (The Conference Secretary, The Institution of Nuclear Engineers, 1 Penderley Road, London SE6 2LQ, UK).

9-13 April, **Residential School on Inorganic Polymers**, London (Ms L.A. Hart, Royal Society of Chemistry, 30 Russell Square, London WC1B 5DT, UK).

10-12 April, **International Conference on Flow Measurement in the Water Industry**, Glasgow, UK (Conference Section, National Engineering Laboratory, Glasgow G75 0QU, UK).

16-19 April, **Annual Chemical Congress of the Royal Society of Chemistry**, University of Exeter, UK (Dr John Gibson, The Royal Society of Chemistry, Burlington House, London W1V 0BN, UK).

17-19 April, **Annual Meeting of the European Society for Clinical Investigation**, Milan, Italy (Organizing Secretariat, Fondazione Giovanni Lorenzini, Via Montenapoleone, 23-20121 Milan, Italy).

29 April-3 May, **Annual Meeting of the American Oil Chemists' Society**, Dallas, Texas (Meetings Coordinator, American Oil Chemists' Society, 508S Sixth Street, Champaign, Illinois 61820, USA).

15-16 May, **International Symposium on Cost and Benefit of Radioimmunoassay**, Milan, Italy (Organizing Secretariat, Fondazione Giovanni Lorenzini, Via Montenapoleone, 23-20121 Milan, Italy).

The **Conference on Hazardous Waste Management** due to take place on 8 December 1983 will now be held on 3 April 1984 at the Sudbury Conference Centre, Newgate Street, London EC1, UK.

The **International Conference on Advances in Surface Coating Technology** which was scheduled to take place in June 1984 has been postponed until 13-15 June 1985.

Students and industrial sponsorship

Richard Pearson*

In certain specialized areas the role of industry in supporting British undergraduates is significant. It can have unexpected consequences.

STUDENT finance has been high on the debating agenda for a number of years. The value of the means-tested grant for British undergraduates is currently £1,660 per annum plus fees although few students receive the full amount because of the means test, with many parents not making their full contribution. Although the real value of the grant was fairly stable throughout the 1970s, it has fallen by about 10 per cent in the past couple of years (Fig. 1). Even so, the British undergraduate is considered better off than his contemporary in Japan, who has to take out a loan, while students in most of Europe and North America have to rely on a combination of loans (often at low rates of interest) and grants, supplemented in some cases by scholarships. In the United Kingdom, the current pressure to reduce public expenditure means that loan

pounds each year.

Sponsorship can take many forms. A student may be a salaried member of the staff of the sponsoring organization, he or she may receive a bursary worth up to £915 a year on top of the mandatory grant, or may have some mix of mandatory grant, bursary and salary when working in vacations or on periods of industrial work if on a sandwich course.

Employers sponsor students for a variety of reasons. For some it is a form of patronage, helping existing employees, often bright craftsmen and technicians, to advance their careers. For most, however, it is a means of guaranteeing a supply of well-trained recruits in the future, particularly in the "shortage" subjects of electronic and mechanical engineering. Sponsorship is also seen as providing an opportunity to make a detailed assessment of an individual's capabilities, as a means of influencing students' choice of subject and institution of study, as a way of attracting high quality school leavers, and to improve collaboration between industry and higher education. There is of course a financial cost involved, up to £4,000 per student over the duration of the course, and there is often the need to provide training and work experience.

In the past, some employers have sought legally to bind the student to the company for a period of years after graduation but this now happens only in the armed forces where the undergraduate becomes an employee. In recent years, 50–80 per cent of sponsored graduates have joined their sponsoring organizations, with each side rejecting the other in roughly similar proportions; some employers are, however, able to retain all those they want. Most students are sponsored from the start of their course, with the majority recruited directly from school, often only from local areas. More than half the employers limit their sponsorship students to particular universities, and in a few cases to particular polytechnics.

Among the 2,200 final-year engineering graduates sponsored in 1983, most were to be found in mechanical (38 per cent) and electrical/electronic (35 per cent) engineering, and relatively few in civil or chemical engineering. While two-thirds were in universities, the proportions of sponsored students, when compared on a subject basis, were similar in the universities and the polytechnics. Overall, 35 per cent of mechanical and 28 per cent of electrical/electronic engineers were sponsored (Fig.

2). It is interesting that it was the largest university/polytechnic departments (with over 50 final year students) that had the highest level of sponsorship and the middle-sized departments (25–49 final year students) that had the lowest.

In the past five years, more than 250 employers have been involved in sponsorship, some with only an occasional student, others with a regular intake of 50 to 100 a year. The number of companies sponsoring students has fallen as training budgets and recruitment levels have been cut back because of the recession. Nevertheless, the number of places on offer has grown slightly, with the number of places for electronic engineers increasing dramatically in response to expected shortages in future years.

Looking to the future, most employers expect to continue with their current sponsorship policies, while one in five expect to increase and one in seven to decrease their level of sponsorship. In the case of electronics, sponsorship has been rising rapidly. As a result, perhaps half of the graduates expecting to enter the labour

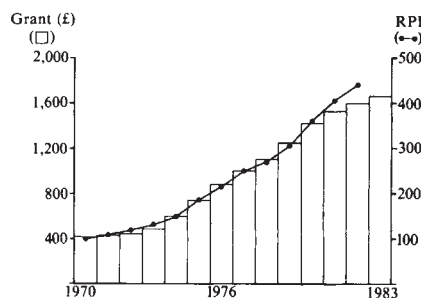


Fig. 1 The "real" value of the student grant in Britain.

schemes could well be reconsidered in the next few years, although opponents argue that loans lead to only limited cost savings, while seriously affecting certain groups, such as women and those from poorer homes.

One source of finance rarely considered in this debate is industrial sponsorship. The full extent, in terms of money spent and numbers involved, is unknown. Most sponsorship is, however, concentrated in the area of engineering, with a smaller involvement in some of the pure sciences and in business and related studies. A recent report¹ from the Institute of Manpower Studies has shown that more than 2,200 (one in four) final-year engineering students were sponsored in 1983, which suggests the total number of sponsored students in higher education is probably over 10,000. Total expenditure by industry is likely to be several million

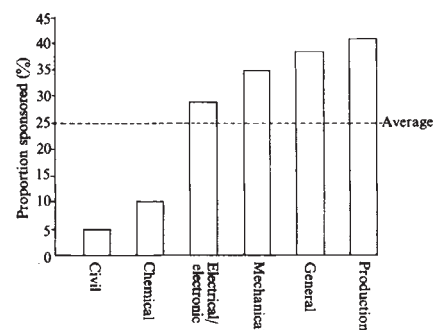


Fig. 2 Proportion of final-year engineering students at UK universities and polytechnics sponsored by an employer during 1983.

market in the next few years will already be notionally committed to a particular employer. While not all will join that employer, it seems that the "free" market of electronics engineering graduates seeking employment will be even smaller than the already inadequate supply than has been forecast. This will compound the future recruitment difficulties of all types of employers, although the sustained high level of sponsorship may have an influence on subject and career choice by would-be students.

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1. Gordon, A., Hutt, R. & Pearson, R. *Undergraduate Sponsorship: Implications for the Labour Market*, Interim Report (IMS, 1983).